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DELIVERY, VIA SURFACE ß-GALACTOSE RECEPTORS, OF LIPOSOMAL 2′,3′-DIDEOXYGUANOSINE-5′-TRIPHOSPHATE TO HUMAN HEPATOMA CELLS TRANSFECTED WITH HEPATITIS B VIRUS

by

YULUN A. HO ©

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA
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Supervisor

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David L.J. Tyrrell

Date July 13, 1994

DEDICATION

To my wife, Meiling Chan and my parents

ABSTRACT

Hepatitis, caused by chronic infections with hepatitis B virus (HBV), is a disease associated with the development of liver carcinoma and cirrhosis resulting in high levels of mortality. We are researching effective methods for delivering antiviral drugs to HBV-infected hepatoma cells (2.2.15 cells). To target hepatoma cells transfected by HBV, liposomes (HSPC:CH 2:1 molar ratio) bearing specific galactose-containing ligands, i.e., liposomal lactosylceramide (L-LAC), asialo-monosialoganglioside (L-ASGM$_1$), and asialofetuin (L-AF) were directed to ß-galactose receptors on the surface of hepatoma cells. It was found that liposomes containing AF (L-AF), conjugated to liposomes by using the biotin-avidin method, resulted in the highest liposomal uptake by 2.2.15 cells. Plain liposomes (ligand-free) resulted in a three-fold lower uptake than that of L-AF. The uptake by 2.2.15 cells of L-LAC or L-ASGM$_1$ were approximately equal and were intermediate between those of L-AF and plain liposomes. A mAb against HBV surface antigen was covalently conjugated on the surface of liposomes and the uptake of these liposomes by 2.2.15 cells was in the same range as the uptake of L-LAC or L-ASGM$_1$.

2′,3′-Dideoxyguanosine (ddG) is a member of the class of dideoxynucleosides which have shown promising results in inhibiting viral replication in several animal models and cell lines. The active form of ddG, the 5′-triphosphate form (ddGTP), is sufficiently hydrophilic to be readily encapsulated in liposomes. It was found that ddGTP had a half life of leakage from L-AF of 6 days at 37°C. To investigate the internalization of liposomes by 2.2.15 cells, HPTS (1-hydroxypyrene-3,6,8,-trisulfonic acid), a pH-sensitive fluorescent compound was entrapped into liposomes (L[HPTS]). By monitoring the emission profiles of L[HPTS], internalization of the liposomes could be monitored. After incubating L-AF[HPTS] with 2.2.15 cells at 37°C for two hours, the pH decreased from pH 7.4 to approximately pH 6.0 indicating the internalization of L-AF into the lysosomal compartment by 2.2.15 cells. An attempt to use L-AF[ddGTP] to inhibit HBV replication in 2.2.15 cells failed. Failure to inhibit HBV replication may result from the acid phosphatase in the lysosomes degrading liposomal ddGTP to ddG, which is inactive in 2.2.15 cells.

In order to determine the intrahepatic distribution of L-AF, a rat model was

used. It was found that the presence of PEG-lipid (polyethyleneglycol-lipid) on the liposomal surface resulted in a longer circulation time than liposomes without PEG. L-AF showed a 30% increased uptake by hepatocytes *in vivo*, as compared to control liposomes lacking AF.

The result of the above experiments indicated that it is possible to deliver liposomal contents into hepatoma cells by targeting to the galactose receptor, and that liposomes targeted to this receptor have increased uptake into hepatocytes *in vivo*.

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ABBREVIATIONS

AF	asialofetuin
AF-liposomes	liposomes conjugated to asialofetuin
AF-PEG-liposomes	liposomes containing polyethylene glycol, conjugated to asialofetuin
$ASGM_1$	asialomonosialoganglioside, GM_1
$ASGM_1$-liposomes	liposomes containing asialomonosialoganglioside, GM_1
biotin-PE	biotinylated phosphatidylethanolamine
CH	cholesterol
ddG	2′,3′-dideoxyguanosine
ddGTP	2′,3′-dideoxyguanosine-5′-triphosphate
DMSO	dimethyl sulfoxide
DSPE	distearoylphosphatidylethanolamine
EDTA	disodium ethylenediamine tetra-acetate
GM_1	monosialoganglioside
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HPTS	1-hydroxypyrene-3,6,8-trisulfonic acid
^{3}H-CHE	[1,2(N)-^{3}H]-cholesterol hexadecyl ether
^{3}H-COE	[^{3}H]-cholesterol oleylether
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid
HSPC	hydrogenated soy phosphatidylcholine
i.m.	intramuscular route
LAC	lactosylceramide
LAC-liposomes	liposomes containing lactosylceramide

LAC-PEG-liposomes	liposomes containing lactosylceramide and polyethylene glycol
mAb	monoclonal antibody
mAb-liposomes	liposomes conjugated to monoclonal antibody against hepatitis B surface antigen
MES	2[N-morpholino]-ethane-sulfonic acid
MPB-PE	N-[4-(p-maleimidophenyl)-butyrly] phosphatidylethanolamine
PEG	polyethylene glycol
PEG-DSPE	poly(ethyleneglycol)-distearoylphosphatidylethanolamine
PL	phospholipid
$T_{1/2}$	half-life
TBS	tris(hydroxymethyl)-buffered saline
TES	N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid
Tris	tris(hydroxymethyl) aminoethane
SDS	sodium dodecyl sulfate
SIgG	sheep IgG

I. INTRODUCTION

(A) *Hepatitis B and Hepatitis B Virus*

(1) Hepatitis

Hepatitis is an inflammation of liver tissue induced by chemicals (e.g. alcohol, some organic solvents, and medication) bacterial or viral infections. It was first described by Hippocrates approximately twenty five hundred years ago. In Germany during the Second World War, more than five million people suffered from hepatitis. This form of hepatitis was formerly called infectious or epidemic hepatitis, but is now referred to as hepatitis A (Lycke and Norrby, 1983).

In 1885 an outbreak of hepatitis was reported among ship-yard workers vaccinated 2 months previously against smallpox. This was the first documented description of hepatitis resulting from a therapeutic measure. It was caused by contamination of the vaccine syringes with blood containing the hepatitis virus. This form of hepatitis, previously termed serum hepatitis, is now called hepatitis B (Gocke and Krugman, 1978; Koff, 1978; Szmuness, 1982).

(2) Hepatitis B virus

The hepatitis-inducing viruses are found throughout the world. In developing countries, hepatitis A is endemic and most individuals are infected at an early age; in industrialized countries hepatitis A is epidemic. Hepatitis B virus (HBV) causes both acute and chronic infections. HBV infection during the neonatal period or early childhood is associated with a prolonged carriership and HBV carriers usually develop chronic hepatitis at times of lowered immunity. There were an estimated 285 million HBV carriers worldwide in 1988 (Di Bisceglie et al., 1988). HBV is an extremely important human pathogen because chronic infection with HBV has been associated with a high risk for development of primary hepatocellular carcinoma (Beasley et al., 1981). The widespread distribution and lethal nature of hepatitis B make the development of treatment for HBV infection especially critical.

HBV is a member of the Hepadnaviridae family, of which several animal hepadnaviruses have been identified. These include woodchuck hepatitis virus

(Summers et al., 1978), ground squirrel hepatitis virus (Marion et al., 1980), and duck hepatitis B virus, DHBV (Mason et al., 1980). Human and animal hepadnaviruses share common biological features including the virion ultrastructure, genomic structure, and a unique mechanism of replication.

HBV has been identified in serum samples of patients with acute hepatitis B, as well as in carriers. Three different kinds of particles are found in serum: spherical (empty, 22 nm in diameter), tubular (empty, 22 nm in diameter; 40-400 nm in length), and Dane particles (with HBV-DNA in the core, 42 nm in diameter). The Dane particles are the true virions of HBV, and the spherical and tubular particles are dummy particles which interfere with the recognition of Dane particles by the immune system. All three kinds of particles are surrounded by a lipoprotein coat (shell) about 7 nm thick. The shell protein on the surface of the particles is referred to as Hepatitis B surface Antigen (HBsAg). There are various subtypes of HBsAg, differentiated by their antigenic specificities. All carry a common determinant called "*a*". The other antigenenic determinants, "*d*", "*y*", "*w*" and "*r*", are present in alternating fashion so that "*d*" excludes "*y*", "*w*" excludes "*r*". Thus four subtypes of HBV can be recognized and defined by the antigenic composition, *adw, adr, ayw*, and *ayr*. Different subtypes of HBV dominate in different regions of the world. Antigens *ayw* are predominant in the USA and northern Europe and are commonly spread by drug addicts. Today this subtype causes increasing numbers of acute hepatitis infections. The antigen complex *adr* is predominant in southern Africa and South-East Asia. It has been introduced to the US by veterans from Vietnam and adopted children immigrating from the Far East or Africa (Lycke and Norrby, 1983).

The Dane particle has an inner core with a diameter of about 27 nm. The core has two specific antigenic structures, Hepatitis B core Antigen (HBcAg), as well as the "*e*" antigen or HBeAg. The core contains a 3250-base circular DNA, which is partly single-stranded, and a specific DNA polymerase.

(B) *Developing Treatments for HBV Infection*

There is no effective antiviral therapy for acute or chronic infection with HBV at present, although several investigators have reported the ability of human interferon and 2′,3′-dideoxynucleosides to inhibit HBV.

(1) Interferon

(a) antiviral therapy with interferon

Interferons are host-derived proteins produced by nucleated cells in response to viruses, and represent an early, pre-antibody, response to viral infection. Interferon can elicit an antiviral response in cells by binding to specific cell receptors and activating intracellular enzymes which activate ribonucleases (Zoon and Arnheiter, 1984). It is suggested that the activation of these ribonucleases, which can destroy viral mRNAs in infected cells, is the primary mechanism by which interferons inhibit viral replication (Davis and Hoofnagle, 1986; Sullard et al., 1981; Lengyel, 1981). Other proposed mechanisms of inhibiting HBV replication by interferons include blockade of virus entry and several immunomodulatory actions (Stewart, 1979). Human interferon preparations have been reported to affect various indices of HBV infection. In some cases of chronic HBsAg-positive hepatitis, interferon was found to reduce HBc-specific DNA-polymerase activity, HBeAg titre, HBcAg titre, anti-HBcAg titre, Dane particle number, and the percentage of liver cell nuclei containing HBcAg. Systemic or local administration (i.m.) of interferons has been tested and shows promising results (Gutterman et al., 1984; Kurzrock et al., 1985).

(b) side effects of interferon therapy

Although interferons have demonstrated clinical value, the usefulness of interferons is limited by their possible toxicity. Chills and fever (38-40°C) were noted in interferon-treated patients after only a single injection. Side-effects were universal during the induction phase and included myalgia, headache, cramps, nausea, and occasionally vomiting. During maintenance therapy, weight loss, fatigue, and loss of concentration were observed. The most serious problem was toxicity to the circulation system. Neutropenia, leucopenia, and thrombocytopenia occurred in most of the interferon-treated patients. In a few cases, hair loss and depression developed

(Alexander et al., 1987; Weimar et al., 1980). The therapeutic benefit of long term treatment also needs to be clarified, since the total disappearance of HBeAg from interferon-treated patients was rarely observed (Alexander et al., 1987). The dosage form necessary to give both a therapeutic effect and reduce adverse side effects remain to be determined.

(2) Dideoxynucleosides (ddNs)

(a) antiviral therapy with ddNs

2′,3′-Dideoxyribosides of the physiological nucleobases (ddNs, Appendix II) and analogs are potent inhibitors of human immunodeficiency virus, an RNA virus (Ahuwalia et al., 1987; Perno et al., 1988). Blockade of viral replication by such agents has been correlated with the intracellular formation of nucleotide intermediates, ddNTPs (triphosphate metabolites), which are presumed to interfere with the synthesis of viral or proviral DNA (Furman et al., 1986; Cooney et al., 1986 and 1987; Starnes and Cheng, 1987; Hao et al., 1988; Busso et al., 1990; Bondoc et al., 1992). It is generally assumed that these compounds have a common mechanism of action, i.e., they are phosphorylated by nucleoside and nucleotide kinases to their respective 5′-triphosphates and, as the latter, act to inhibit retroviral reverse transcriptase, thus showing the mis-incorporation of physiological 2′-deoxyribonucleotides into viral DNA (Cheng et al., 1987).

A second possible mechanism is that ddNs themselves can serve as the initial terminator of DNA synthesis. It is thought that ddNs, as alternate substrates, can react with the viral DNA polymerase and become incorporated into the growing DNA sequence, thus elongating the chain by one residue and terminating further DNA synthesis (Mitsuya et al., 1987). This proposed mechanism expands the use of ddNs into the field of DNA virus infections. Studies have shown that ddNs and their analogs have the potential of inhibiting HBV replication by the same mechanism mentioned above (Weller et al., 1982; Perrillo et al., 1985; Lee et al., 1989; Chang et al., 1992). Several attempts at using ddNs and analogs to treat human HBV or duck HBV (DHBV) infections have given exciting results. Among the ddNs and their analogs, (-)-2′,3′-dideoxy-3′-thiacytidine ((-)-3TC, Appendix II) was found to be the most potent inhibitor of HBV DNA replication, with an ID_{50} of 0.05 μM as monitored by the amount of both intracellular and secreted viral DNAs in an HBV-transfected human hepatoma cell line, i.e., 2.2.15 cell line. It was found that (-)-3TC could also inhibit the replication of DHBV in a Peking duck model (Chang et al., 1992). This result further supported the possibility of using (-)-3TC as an anti-HBV drug. This drug is now in phase II clinical trials in humans.

(b) side effects of ddN therapy

The possible toxicity of ddNs and their analogs is still a major obstacle for using these antiviral drugs, particularly in long-term-therapy. This concern is a result of problems encountered in a clinical study which used single or double cycles of an adenine analog, adenine arabinoside (araA), to treat HBV infection (Weller et al., 1982; Perrillo et al., 1985). It was found that nausea occurred in all patients in these clinical trials. The most troublesome side effect was a peculiar neuromuscular pain syndrome which occurred in 70-80% of patients treated with araA. These side effects were thought to be caused by the specific mode of action of the nucleoside analogs. Since the nucleoside analogs are acting mainly by interfering with the metabolic pathway of nucleotide synthesis, it is thought that ddNs and their analogs might induce similar side effects by competing in the nucleoside metabolic pathway.

2′,3′-dideoxycytidine (ddC) is the most broadly studied member of ddNs. It was found that ddC resulted in neurotoxicity in rabbits (Feldman and Anderson, 1994). Rabbits received an oral treatment with 35 mg/kg per day of ddC for 16 days developed mitochondrial alterations in the Schwann cells of sciatic and tibial nerves and of dorsal root ganglia, and were positively correlated with myelin pathology, e.g., myelin splitting, intramyelinic edema, and demyelination of the largest diameter myelin sheaths in the proximal nerve segments of the sciatic and ventral roots. 2′,3′-Dideoxyinosine has been related with peripheral neuropathy and pancreatitis (Keilbaugh et al., 1993). For deoxynucleoside derivatives, the famous drug, 3′-azido-

3′-deoxythymidine or AZT, resulted in bone marrow suppression, myopathy, and cardiomyopathy; 3′-deoxythymidine-2′-ene was associated with pancreatitis. In the case of clinical trial of fialuridine (FIAU), the long-term hepatic toxicity of FIAU, which was not revealed in animal studies, resulted in a tragedy with the death from liver and pancreatic damage of five HBV-infected patient enrolled in a six-month course of FIAU treatment (Marshall, 1994). The long-term toxicities of ddNs, and analogs of ddNs are now related to the possible damage to mitochondrial DNA resulted from the treatments of nucleoside drugs (Marshall, 1994).

(3) Development of Improved Treatments for HBV Infection

When developing new anti-HBV therapeutics, one must consider how to prevent possible toxicity of ddNs, ddN analogs and interferons to normal cells and how to increase the specific toxicity of these antiviral agents to HBV-infected cells. A drug delivery system can carry drugs to infected cells specifically, thereby decreasing the toxicity of the drugs to normal tissues. Liposomes are thought to be excellent candidates for use as drug carriers. Encapsulation of antiviral agents into liposomes may resolve the problem of non-specific toxicity.

(C) *Liposomes and Their Value in Clinical Therapy*

Liposomes were first described about 30 years ago by Bangham and Horne (1964), who observed that addition of aqueous media to dried phospholipid films deposited on the wall of glass containers resulted in the spontaneous formation of phospholipid bilayers. Simple agitation caused the formation of structures that had several concentric bilayers of phospholipids, and these were called multilamellar vesicles (MLVs) to reflect their physical structure.

It soon became apparent that biologically active substances trapped within liposomes may be treated differently by cells and tissues than these same substances in their free form. As a result, liposomes have become popular tools for biochemists, cell physiologists, and investigators interested in drug delivery systems (Gregoriadis, 1973).

(1) Conventional Liposomes and Their Disadvantages

In the particular field of drug delivery, liposomes were criticised as a pharmaceutically unacceptable preparation, because of their unstable composition, inability to entrap amphipathic drugs, and the expensive cost of production. After years of effort, investigators have overcome some of these problems. With the improvement of biochemical technology, pure phospholipids in large quantity can be obtained. Improved liposomal formulations make liposomes stable enough to prevent the leakage of entrapped drugs and decrease the decomposition of the liposomes themselves.

Even so, the utility of liposomes as drug carriers *in vivo* is still limited by their short residence time in circulation. Conventional liposomes, or C-liposomes (e.g. egg phosphatidylcholine : cholesterol 2:1 molar ratio), given intravenously, are cleared from blood in 2-4 hours in a mouse model (Allen and Chonn, 1987; Allen et al., 1989). This short lifespan is due to the fast clearance of liposomes by the mononuclear phagocyte system (MPS), also termed the reticuloendothelial system. The MPS consists of circulating macrophages and resident macrophages in liver (Kupffer cells), spleen, bone marrow, lymph nodes and lungs (Allen et al., 1984; 1989). When liposomes are introduced into the circulation, they rapidly accumulate in the MPS, particularly in the liver and spleen. Clinical applications of liposomes in tissues other than the MPS will only be feasible when a method of avoiding uptake by the MPS is developed.

(2) Long-Circulating Liposomes

Liposomes which remain in circulation longer would have more likelihood of reaching non-MPS sides of disease. When glycolipids were added to the liposome formulation, successful avoidance of the MPS was accomplished. In 1987, long circulating liposomes were first reported by Allen and coworkers (Allen and Chonn, 1987; Allen et al., 1989). They found that liposomes containing monosialoganglioside, GM_1, in addition to egg phosphatidylcholine and cholesterol, had twice the lifespan of C-liposomes. It was found that up to 60% of GM_1-liposomes were still in circulation 24 hours after injection (Allen and Chonn, 1987; Allen, 1992; also Gabizon and Papahadjopoulos, 1988). This advance permitted the development of several clinical applications for liposomes.

Later investigators discovered that incorporation of polymers, such as polyethylene glycol (PEG)-lipid derivatives into liposomes also result in long circulating liposomes (Allen et al., 1991a; Woodle and Lasic, 1992; Senior et al., 1991). Liposomes containing GM_1 or PEG-lipids have increased surface hydrophilicity and decreased opsonisation by plasma proteins, leading to decreased recognition and uptake by MPS (Wassef et al., 1991; Chonn and Cullis, 1992; Senior, 1992; Alving and Wassef, 1992; Gabizon and Papahadjopoulos, 1992).

(a) mechanisms of long-circulating effect of liposomes containing monosialoganglioside (GM_1)

The existence of screened negative charges on GM_1 is thought to increase the surface hydrophilicity of the liposomes without presenting a negatively charged binding site at the surface of the liposome (Allen et al., 1989 and Allen, 1994a; Gabizon and Papahadjopoulos, 1992). A second mechanism to account for the longer half-life of GM_1-liposomes is based on the observation that decreased opsonization by plasma proteins is in part dependent on bilayer rigidity. Sphingomyelin (SM), which increases the rigidity of liposomes, prolongs the lifespan of GM_1-liposomes (Allen and Chonn, 1987; Allen et al., 1994a). Incorporation of SM into GM_1-liposomes may also make liposomes mimic the outer surface of red blood cells and avoid macrophage uptake (Allen et al., 1988).

(b) mechanisms of long-circulating effect of liposomes containing polyethylene glycol lipid (PEG-lipid)

Liposomes containing PEG-lipid (PEG-liposomes) have increased surface hydrophilicity due to the polarity of PEG itself (Illum et al., 1986; Allen et al., 1989, 1990, 1991a and 1991b; Senior et al., 1991; Mori et al., 1991; Chonn and Cullis, 1992; Lasic et al., 1992; Woodle and Lasic, 1992). Every ether oxygen group in PEG can form a hydrogen bond with two molecules of water (Martin et al., 1983). These two bound water molecules will capture other water molecules and finally form a water coating on the liposomal surface. Increased hydrophilicity and rapid vibration of the exposed PEG-chains can prevent binding of plasma proteins and make the liposome surface resemble water. By this means, PEG-liposomes can escape uptake by the MPS. The water-like structure conferred on the liposome surface by PEG can also reduce or eliminate any electrostatic interaction between liposomes and plasma ions, such as calcium, sodium and phosphate. This may decrease the opsonization of the liposomes by plasma proteins, whose attachment to cell membrane or liposomal surface is ion-dependent. PEG on the liposome surface may also make a more tightly packed bilayer with strong intrabilayer attractive forces, which can greatly reduce the anchoring, adhesion or adsorption of plasma proteins to the liposomes in the absence of electrostatic forces, despite hydrophobic and Van der Waals forces favouring such interactions (for review: Woodle and Lasic, 1992). The term, "sterically stabilized liposomes" or "Stealth™ liposomes" (S-liposomes) was adopted to represent liposomes containing PEG-lipid, because the stabilized liposomal surface makes liposomes avoid capture by the MPS.

Liposomes containing glycolipid are abbreviated as LC-liposomes (long circulating liposomes). Both LC-liposomes and S-liposomes offer the possibility of developing practical liposomal delivery systems. LC- or S-liposomes are eventually removed from the circulation by the MPS, and the liver and spleen are still the major organs of accumulation (Allen et al., 1989; Woodle et al., 1990; Liu et al., 1991; Huang et al., 1992; Bakker-Wouderberg et al., 1992).

(3) Liposomal Targeting

(a) passive and active targeting

Once long-circulating liposomes have been developed, targeting LC- or S-liposomes to specific diseased cells is the next barrier to overcome. Since the MPS accumulates liposomes rapidly and actively, it is reasonable to target to the MPS and treat MPS-related diseases by applying liposome delivery systems. An attempt to use liposomes to treat leishmaniasis was the earliest report of targeted liposome therapy (Alving et al., 1978).

The uptake of LC- or S-liposomes into solid tumours, or infected tissues, is increased compared to C-liposomes, primarily due to the increased circulation half-life of the LC- or S-liposomes. Thus they are able to access the diseased tissues, which have increased capillary permeability (Gabizon and Papahadjopoulos, 1988; Huang et al., 1992; and Zou et al., 1993). This so-called passive targeting to diseased tissues can increase local drug concentrations and thereby decrease side effects of systemic therapy, thus increasing therapeutic efficacy (for review: Allen, 1994a,b). The term active targeting has been used to refer to ligand-mediated targeting. In active targeting, a ligand which binds to a specific molecule on the targeted cell can be attached to the surface of the liposome, promoting specific binding of the liposome to the targeted cell. Active targeting of LC- or S-liposomes has been shown to increase localization of liposomal drugs in diseased tissues, and increase therapeutic effects (Ahmad et al., 1993; Zou et al., 1993; Allen, 1994b).

(b) targeting liposomes to hepatocytes: passive or active targeting?

As mentioned above, the major problem in developing anti-HBV agents is how to increase their selective toxicity to diseased cells while decreasing their non-specific toxicity to normal cells. Liposomal encapsulation of drugs can dramatically alter the distribution of drugs in the body and the rate of clearance (Allen and Hansen, 1991). These pharmacokinetic differences and other less well understood effects, can result in passive targeting of a drug to the liver, prolonged blood levels or tissue levels of drug, reduced toxicity, and enhanced efficacy of the encapsulated drugs. These advantages of a targeted liposomal delivery system may result in improved therapy for HBV.

From the viewpoint of efficacy, passive targeting may not be an effective method for HBV therapy. Although liposomes are accumulated in the liver passively, they are mainly taken up by Kupffer cells rather than hepatocytes, the host cells infected by HBV. Decreasing liposome size (to less than 90 nm in diameter) can improve uptake by hepatocytes relative to Kupffer cells (Alino et al., 1993; Scherphof et al., 1983). This is because small liposomes can pass through hepatic sinusoid pores (100-150 nm in diameter, Meijer et al., 1992; Meijer and Ziegler, 1993) to escape capture by Kupffer cells. However, with decreased liposome size, the efficiency of entrapment of drugs decreases. Since most of the experimental drugs are expensive and difficult to produce, a low entrapment efficiency is undesirable. Larger liposomes (90-200 nm) give better entrapment efficiency, as compared to small liposomes, and are mainly accumulated in liver (Allen and Everst, 1983; Allen et al., 1988 and 1991b; Liu et al., 1992; Allen and Hansen, 1991). Thus for the treatment of HBV infections, active targeting of liposomes with an average diameter of 90-100 nm seems a better choice. In addition, ligand-mediated targeting should optimize the specific uptake of liposomes by the target hepatocytes.

(c) ß-galactose receptors: possible receptors for targeting hepatocytes

(i) properties and functions of ß-galactose receptors

The ß-galactose receptor, found on the parenchyma cells of the mammalian liver, is a potential target for active targeting of liposomes to hepatocytes (Wall et al., 1980; Ashwell and Harford, 1982). This specific receptor is proposed as the receptor for regulation of plasma glycoproteins. The discovery of the ß-galactose receptor came from research into the fate of glycoproteins in plasma. Early studies showed that removal of a sialic acid residue from glycoproteins caused rapid clearance of glycoproteins by liver (Morell et al., 1971). For this reason, it is believed that a sialic acid residue is essential for glycoproteins to stay in circulation (Hickman and Ashwell, 1970).

Further investigations showed that loss of a sialic acid residue resulted in the exposure of a galactosyl group, which could function as the ligand recognized by a specific receptor on the surface of hepatocytes (Gregoriadis et al., 1970). This specific receptor on hepatocytes was first demonstrated by using radiolabelled desialylated ceruloplasmin. By tracing the radioactivity of asialoceruloplasmin, the ß-galactose receptor was located. It was found that injected asialoceruloplasmin disappeared from circulation rapidly. In a rat model, approximately 98% of the total radioactivity was taken up by the liver within 15 min. When unlabelled asialoceruloplasmin was injected simultaneously, less than 4% of the radioactivity was found in liver. These results indicate that the ß-galactose receptors were found mainly in the liver and they would specifically bind to an exposed galactose group on glycoproteins. Furthermore, all of the radioactivity recovered in the liver was seen to be present in the parenchymal cells, none was observed in the Kupffer cells (Morell et al., 1968; Hickman and Ashwell, 1970). The lack of cross-inhibition of hepatic uptake between the asialo-plasma proteins and a readily phagocytosed colloid confirmed that Kupffer cells did not participate in the uptake of asialoceruloplasmin (Ashwell and Harford, 1982).

In a series of experiments done on rabbits (Morell et al., 1966), it was found that 90% of injected asialoceruloplasmin was cleared from circulation within 15 min, whereas only 10% of native ceruloplasmin was removed from blood. Increased clearance of asialoceruloplasmin could be reversed by treating asialoceruloplasmin with galactose oxidase before injection. This converted 94% of the primary carbonyl groups of galactose residues to aldehyde groups, which could not be recognized by the ß-galactose receptor (Morell et al., 1966). A specific structure of asialo-molecules may also be involved.

It was found that there was a variation in the affinities of different ligands for the receptors. The spectrum of activities for binding of serum proteins is bounded by asialo-orosomucoid (high affinity) and asialotransferrin (low affinity) (Ashwell and Hickman, 1970; Regoeczi et al., 1974). Inspection of the carbohydrate content and structure of these ligands reveals that orosomucoid contains a five branched chain carbohydrate unit that is asymmetrically arranged on the polypeptide near the amino terminus, and that transferrin, for the most part, contains only biantennary type glycose chains (Ashwell and Hickman, 1970 and Kawasaki and Ashwell, 1976).

Arrangement of glycose residues, as well as branch number on the sugar chain, has been investigated. Baenziger and Maynard (1980) have examined in detail the interaction of asialotransferrin with the hepatic binding protein. They found that while individual asialotransferrin molecules are readily bound by the receptor, they are not endocytosed. To explain this observation, it was proposed that simultaneous binding at two receptor sites 25-30 Å apart, rather than binding at a single site, was required for high affinity binding. This general picture is supported by the finding that as increasing amounts of galactose are added to synthetic substrate, a threshold for binding to rat hepatocytes is observed (Weigel et al., 1979; Ashwell and Hartford, 1982). This can best be explained in terms of a critical density being required for appropriate spacing of sugars.

A transmembrane structure was proposed for the ß-galactose receptor. By using goat antibodies against the isolated ß-galactose receptor from rat liver, it was found that this anti-receptor antibody bound not only to the perfused rat hepatocytes but also bound to hepatocyte plasma membranes oriented with their cytoplasmic surface outward on polylysine-derivatized beads (Harford and Ashwell, 1981). This discovery confirmed that the ß-galactose receptor is a member of the transmembrane protein family.

ß-galactose receptors are clustered together in the coated pits located on hepatocytes (Ashwell and Harford, 1982). Wall and coworkers (1980) used two electron microscopic tracers, asialo-orosomucoid covalently coupled to horseradish peroxidase (ASOR-HRP), and lactosaminated ferritin (LAC-Fer), to investigate the internalization of asialoproteins bound by the ß-galactose receptors of rat hepatocytes. Thirty seconds to two minutes after injection, the tracers began to accumulate on the periphery of the cell where coated pits are located. Five minutes after injection, the tracer began to appear in Golgi-lysosome regions of the hepatocyte. Some of the LAC-Fer-containing vesicles located in Golgi-lysosome areas 15 minutes after injection were found to contain aryl sulfatase reaction product, indicating fusion with lysosomes (Gregoriadis et al., 1970; Tanabe et al., 1979; Bridges et al., 1982). It was concluded that asialo-molecules were internalized by hepatocytes, and the lysosome was the probable organelle in which the receptor-ligand complex was degraded.

The properties of the ß-galactose receptor have been broadly investigated. The ß-galactose receptor of rat liver has a turnover rate of approximately 20 hours, and the dissociation constant for the single high affinity binding site was calculated to be 3.4×10^{-8} M (Steer and Ashwell, 1980). The inhibition constant measured by using ^{125}I-asialo-orosomucoid to compete with the binding between asialofetuin and ß-galactose receptor was found to be 1.7×10^{-8} M (Baenziger and Maynard, 1980). Additionally, other kinetic parameters, i.e., association rate constant, and the internalization rate constant, were determined (Tolleshaug, 1981; Harford et al., 1983). Based on the estimates of the metabolism rate of the asialoglycoprotein ligands relative to the turnover rate of the receptor, it was concluded by Doyle and Warren (1981) that each molecule of receptor can deliver about 1,000 molecules of ligand to the lysosome to be degraded. This cyclic use of the ß-galactose receptor was supported by several pieces of evidence. One of the most convincing was that, even when the synthesis of receptor was blocked by 1 mM cycloheximide, 34 times more asialo-orosomucoid was metabolized than could be bound by the cell surface receptors, or twice the total capacity of the intact hepatocytes (Steer and Ashwell, 1980).

The rapid binding of ligands, the excellent capacity of internalization, and the specific location on parenchyma cells make the ß-galactose receptor a good receptor for targeting liposomes for the treatment of HBV.

(ii) ß-galactose receptor-mediated liposomal targeting to hepatocytes

In the 1970's, the possibility that the ß-galactose receptor might be a good candidate for liposomal targeting was investigated by several groups. Ghosh and Bachhawat (1980) inserted asiaologangliosides and ß-galactose onto the surface of radiolabelled liposomes by mixing asialogangliosides (or ß-galactose) with phospholipids. They successfully demonstrated that liposomes with surface asialogangliosides or ß-galactose were rapidly taken up by the liver. This uptake was blocked by asialofetuin, a triantennary asialoprotein. These results suggest that the uptake of asialo-liposomes is through the same receptor that takes up asialofetuin. This investigation also found that, at 15 minutes post-injection, the lysosomal-mitochondrial fraction of homogenated hepatocytes contained about 30-40% of the total radioactivity recovered in the homogenate, indicating the internalization of liposomes incorporated with asialo-molecule. Even when the liposomal uptake was blocked by asialofetuin, the subcellular localization of the radioactive marker associating with cells was not affected, i.e. there was still 30-40% of recovered radioactivity in the lysosomal-mitochondrial fraction. This indicated that the intracellular distribution of internalized asialo-liposomes was not determined by the quantity of material internalized.

The uptake of asialoganglioside-liposomes undergoes a significant variation with the phospholipid/glycolipid ratio. There was no galactose-specific uptake of these liposomes at an asialoganglioside content of less than 3 mol% (Ghosh et al.,1982; Ghosh and Bachhawwat, 1982). The uptake of these liposomes by liver increases with increasing content of asialogangliosides on the liposomes surface. This suggests that the uptake of asialoganglioside-liposomes is a function of the surface density of glycolipids, supporting the theory that a critical density of ligand is necessary for recognition by receptors. This phenomena of requiring threshold concentration of ligands on liposomes had also been reported for lactosylcerebroside liposomes (Szoka and Mayhew, 1983).

A subsequent experiment, done by the group of Ghosh (1982), demonstrated that asialoganglioside liposomes were specifically taken up by parenchymal cells and liposomes containing mannose were recognized by another hepatic carbohydrate-specific receptor, the N-acetyl-glucosamine receptor, specifically located on the surface of Kupffer cell and monophagocytes. Thus, the specific recognition of asialo-gangliosides or ß-galactose by the ß-galactose receptor was not altered by incorporating them into liposomes (Dasgupta and Bachhawat, 1985). This observation further supported the use of the ß-galactose receptor as a target directing liposomes to parenchymal cells (Ghosh and Bachhawat, 1992).

(4) Liposomal Encapsulation of Interferon and Delivery, via ß-Galactose Receptors, to Hepatoma Cells Transfected with HBV

Recently, a series of experiments done by Tsuchiya and coworkers (1990 and 1991) have demonstrated that interferon-γ encapsulated into ß-galactose-receptor-targeted liposomes results in a higher therapeutic effect on HBV infection than untargeted liposomes. It was found that asialofetuin-incorporated liposomes increased the internalization of encapsulated interferon-γ via the specific ß-galactose receptor on the surface of infected cells. These experiments provided much information about targeting liposomes to the ß-galactose receptors and showed that an increased uptake of antiviral agents by HBV-infected hepatocytes could be obtained by using ß-galactose-receptor-targeted liposomes containing the encapsulated drug. However, the advantage of receptor-targeted liposomes is arguable, since free interferon-γ showed the most effective inhibition of HBV replication in all cases (Tsuchiya et al., 1990; 1991).

(5) Liposomal Encapsulation of ddNTPs and Delivery to Macrophages Transfected with Human Immunodeficiency Virus (HIV)

The encapsulation of antiviral drugs, e.g., ddNs, into liposomes has been demonstrated by several investigators (Kim et al., 1990; Szebeni et al., 1990; Betageri, 1993; Betageri et al., 1993; Mirchandani and Chien, 1993). It was found that the ddNTPs, which are much more hydrophillic than ddNs, were fairly stable after encapsulation. The leak of ddNTPs from liposomes can be controlled by using rigid liposomes (i.e., containing cholesterol) instead of fluid liposomes (Szebeni et al., 1990; Betageri, 1993; Betageri et al., 1993). The ability to inhibit HIV replication by encapsulated ddNTPS has been examined in monocyte/macrophage cultures using untargeted liposomes. It was suggested that the use of liposomes for targeting drugs to macrophages *in vivo* could be exploited to improve the therapeutic index of ddNs. To date, there is still no report about active targeting of ddNs, their analogs, or the active ddNTPs, encapsulated in liposomes, to hepatocytes.

(D) *Purine ddNTPs as Potential Liposome-dependent Antiviral Drugs*

ddNs have been suggested to be a useful group of antiviral agents. Even though all ddNs have several similarities, it has been observed that the sub-groups of ddNs, purine and pyrimidine ddNs (Appendix II), show different potency against mammalian HBV and avian HBV (Hao et al., 1988; Lee et al., 1989; Busso et al., 1990; Bondoc et al., 1992). It was found that purine ddNs block DHBV replication at very low concentrations, whereas the pyrimidine ddNs (with the exception of dideoxycytidine) have little effect. In contrast, pyrimidine ddNs show a stronger therapeutic effect in inhibiting mammalian virus than do purine ddNs. After injection, purine ddNs reach only a very low plasma concentration of active form (ddNTPs) in mammalian models. The same amount of pyrimidine ddNs reached a higher plasma concentration of the active form (Lee et al., 1989; Kitos, 1994).

Further investigation (Kitos, 1994) revealed that purine ddNs did not show any therapeutic effect on the HBV-transfected human hepatoma cell line (2.2.15 cells), and the active triphosphate metabolites of purine ddNs were undetectable in these cells. In contrast, pyrimidine ddNs could inhibit the HBV replication in 2.2.15 cells, and pyrimidine ddNTPs were the major metabolites in the transfected cells. It is proposed that the concentration difference between metabolized active forms of purine and pyrimidine ddNs may be involved in the differential expression of antiviral efficacy. One proposed explanation is that the specific kinases which can convert pyrimidine ddNs into pyrimidine ddNTPs may predominate in mammalian cells. In contrast, purine ddNs kinases may predominate in avian cells. These phenomena suggest that direct delivery of purine ddNTPs into mammalian cells, rather than sending the prodrugs, ddNs, may overcome the disadvantage of applying purine ddNs, i.e., dideoxyguanosine (ddG), dideoxy-inosine (ddI) and dideoxy-adenosine (ddA), to mammals. Our special interest in ddGTP is thus due to its potential for becoming a liposome-dependent antiviral agent.

(E) *Thesis Objectives*

This thesis will explore the possibility of using actively targeted liposomes with entrapped ddNTPs, particularly 2',3,-dideoxyguanosine-5'-triphosphate or ddGTP, to inhibit HBV replication in hepatoma cells. The main approach to active targeting of liposomes to hepatoma cells will be to direct liposomes to the ß-galactose receptors on both transfected and non-transfected hepatoma cell lines. The possibility of using immunoliposomes against HBsAg to deliver ddGTP to HBV-infected hepatocytes will also be studied. Uptake phenomena, internalization of targeted liposomes, and the effect of inhibition of HBV replication are the major topics which will be studied in this thesis. The specific objectives of this thesis are:

(1) To conjugate the mAb against HBsAg to liposomes by using a covalent binding method and examine the specific uptake behaviour of these immunoliposomes in both a human hepatoma cell line and a HBV-transfected human hepatoma cell line.

(2) To conjugate or incorporate three different ß-galactose receptor ligands (asialofetuin, asialo-GM_1, and lactosylceramide) to liposomes and examine the specific uptake of each type of ligand-liposome in both a human hepatoma cell line and a HBV-transfected human hepatoma cell line.

(3) To encapsulate the antiviral drug, ddGTP, into various liposomes and investigate the ratio, efficiency of drug entrapment and leakage of drug from liposomes.

(4) To examine the internalization of asialofetuin-conjugated liposomes by a HBV-transfected human hepatoma cell line.

(5) To investigate the therapeutic efficacy of liposome-encapsulated ddGTP in a HBV-transfected human hepatoma cell line by monitoring the inhibition of HBV replication.

(6) To examine the uptake by liver of liposomes conjugated to asialofetuin (with or without PEG) and the specific intrahepatic distribution of various liposomes into parenchymal cells or Kupffer cells in rats.

II. MATERIALS AND METHODS

(A) *Materials*

Hydrogenated soy phosphatidylcholine (HSPC) was a generous gift from Liposome Technology, Inc. (Menlo Park, CA) and cholesterol (CH) was purchased from Sigma (St. Louis, MO). Alpha-MEM (Minimum Essential Media) culture media, trypsin, Geneticin™ and Pen-Strep™ were purchased from Gibco Canada (Burlington, ON). Fetal bovine serum (FBS) was from Flow USA (McLean, VA). [1,2(N)-^{3}H]-Cholesterol hexadecylether ([^{3}H]CHE) and 2′-deoxyguanosine-triphosphate (^{32}P-labelled) (^{32}P-dGTP) were purchased from Dupont Canada (Mississauga, ON). ^{125}I was obtained from Amersham Canada (Oakville, ONT). The ß-galactose-receptor ligands, lactosylceramide, asialo-ganglioside-GM$_1$ (Asialo-GM$_1$), and asialofetuin were from Sigma (St. Louis, MO). Purified mouse anti-human hepatitis B virus surface antigen (HBsAg ad+ay subtypes) specific monoclonal antibody (mAb against HBsAg) was purchased from CedarLane Laboratories (Hornby, ONT). 2′,3′-Dideoxy-guanosine-triphosphate was synthesized by Dr. J. Wilson (Department of Medical Microbiology and Infectious Diseases, University of Alberta, Canada). Avidin (egg) was purchased from Sigma (St. Louis, MO) and biotinylated phosphatidylethanolamine (biotin-PE) was obtained from Avanti Polar Lipids (Birmingham, AL). N-Hydroxysuccinimide biotin (NHS-Biotin) and Iodobeads™ were from PIERCE US (Rockford, IL). N-Succinimidyl pyridyl dithiopropionate (SPDP), N-[4-(p-maleimidophenyl)-butyryl]phosphatidylethanolamine (MPB-PE), dithiothreitol (DTT), and related agents were purchased from Sigma (St. Louis, MO). Polycarbonate filtration membranes, Nucleopore™ (pore size: 0.1 μm and 0.2 μm), were obtained from Costar (Cambridge, MA). For *in vivo* studies, poly(ethyleneglycol)-distearoylphosphatidylethanolamine (PEG-DSPE), molecular weight 1900, was a generous gift of Liposome Technology, Inc., Menlo Park, CA. Collagenase for liver cell isolations were purchased from Sigma. Nycoden™ for the flotation method of purifying cell fractions was from Nycomed (Norway). All other chemical materials used in experiments were reagent grade.

(B) *Liposomes*

(1) Preparation of Liposomes

Multilamellar vesicles (MLVs) composed of HSPC:CH with a molar ratio of 2:1 were formed first. At room temperature (23-25°C), the desired amounts of phospholipid and cholesterol stock solutions (in chloroform) were mixed and dried under rotary vacuum to form a thin film of lipids which was then rehydrated by adding 1 or 1.5 ml of HEPES buffer (25 mM HEPES, 150 mM NaCl, pH 7.4). After vigorous vortexing for one minute at least three times and, occasionally, brief sonication for less than five seconds (two or three times), rehydrated phospholipid spontaneously formed MLVs. MLVs were subsequently extruded (Extruder™, Lipex Biomembranes, Vancouver, BC) at 60°C (above the phospholipid phase transition) through two stacked 0.2 μm Nucleopore polycarbonate filters at least ten times, by using compressed nitrogen at approximately 100 p.s.i. This was followed by more than ten times of extrusion by passing through two layers of 0.1 μm filters at 200-400 p.s.i., to make liposomes with an average diameter of approximately 100 nm. Liposomal size was routinely measured at room temperature by dynamic light scattering using a Brookhaven B190 particle sizer (Brookhaven Instrument Corp., Holtsville, NY).

Sometimes, a tracer amount of $[^3H]$CHE (1 μCi/8-10 μmol of phospholipid), an *in vitro/vivo* non-exchangeable, non-metabolizable material, was added into the phospholipid/cholesterol mixture during the formation of liposomes to serve as a marker for measuring liposome uptake. The concentration of phospholipid was determined by the Bartlett method described previously (Bartlett, 1959). In brief, suitable amounts of sampled liposomes and 100 μl standard samples (1 mM KH_2PO_4 in double distilled water) were digested in acid-washed tubes by heating them with 500 μl of perchloric acid (10 N) for each sample on bunsen burner. Tubes were shaken continuously to prevent sudden expulsion of contents. Samples were kept on flame and color change of colorlessness into light brown and turning again into colorlessness indicated the completion of digestion. With vortex mixing, 4.6 ml of 0.22% ammonium molybdate and 200 μl of Fiske SubbaRow reagent (Appendix I) were added into each sample, sequentially. Samples were then heated in a boiling water bath for 7-14 min. After cooling down, the absorbency of each sample was read at 830 nm in a spectrophotometer (Beckman DU Model-2400). The phospholipid concentration of each sample was calculated with reference to a standard which contained 0.1 μmoles of phosphate.

(2) Cell Lines

Human hepatoma cells (HepG2 cells) and HepG2 cells transfected with human HBV (2.2.15 cells) were utilized as experimental models (Acs et al., 1987; Sells et al., 1987; 1988). Hepatoma cells, both non-transfected and transfected, were grown in MEM medium with 10% of fetal bovine serum, FBS (MEM supplemented with 750 mg/L sodium bicarbonate, 300 mg/L L-glutamine). In the case of the HepG2 cell line, Pen-Strep™ was added into the MEM medium to prevent the possible contamination of bacteria (50 IU/ml penicillin G, 10 μg/ml streptomycin sulphate). For the 2.2.15 cell line, Geneticin™ (380 mg of geneticin sulphate/L MEM) was used instead of Pen-Strep™, because transfected cells are more resistant to Geneticin™ than are HepG2 cells. This is because the vector used to transfect HepG2 cells contains not only an HBV-DNA fragment but may also a marker which confers the resistance to Geneticin™ (Sells et al., 1987; Moriarty et al., 1981). Cells were kept in an incubator, at 37°C, in a humidified atmosphere of 5% CO_2 in air, and grown as monolayers.

(3) Coupling of Ligands on Liposomes

For "targeting" the liposomes to hepatomas, two methods were used. In one method, we conjugated mAb against HBsAg on the surface of liposomes (mAb-liposomes) and measured the uptake of mAb-liposomes by both infected and non-infected hepatoma cells. MPB-PE was incorporated into liposomes during their formation (HSPC:CH:MPB-PE 10:5:0.1 molar ratio) to serve as the anchor for coupling mAb. Liposomes containing MPB-PE were produced as mentioned above and the average size was controlled at approximately 100 nm in diameter. By incubating mAb against HBsAg (1 mg/ml of HEPES saline buffer, 25 mM HEPES, 150 mM NaCl, pH 7.4) with 25 mM SPDP (7.8 mg/ml ethanol), at a SPDP:mAb 10:1 molar ratio, at room temperature for 30 min., the pyridyl dithiopropionate (PDP) group was coupled to the mAb through its -NH_2 group. Free SPDP was separated from PDP-mAb by passing through a Sephedex G-50 column (1x20 cm), eluting with 100 mM sodium acetate, 100 mM NaCl, pH 4.7 buffer. The protein peak was determined from the absorbency at 280 nm of eluted fractions and the final concentration of pooled mAb was measured by the BioRad™ protein assay (BioRad, USA). A thiol group was generated on the PDP-mAb by incubating with 2.5 M DTT (385 mg/ml 100 mM sodium acetate, 100 mM NaCl, pH 4.7) at a ratio of 10 μl DTT/ml mAb (final concentration: 25 μmol DTT/ml mAb) for 30 min at room temperature. This resulted in the formation of the -SH group, a much stronger nucleophilic group than -NH_2. By passing through a Sephadex G-50 column (1x30 cm) with 25 mM HEPES, 25 mM MES, 150 mM NaCl, pH 6.7 buffer, non-reacted DTT was separated from thiolated mAb. The protein peak was also determined from the absorbancy at 280 nm of eluted fractions and the BioRad method was used to estimate the final concentration of pooled mAb solution which was further diluted to 1 mg of mAb/ml with 25 mM HEPES, 25 mM MES, 150 mM NaCl, pH 6.7.

A covalent thiol ether bond was established between the -SH group of thiolated mAb and the maleimidophenyl group of MPB-PE, contained in the liposomes, by incubating them together (100 μg of mAb/μmol phospholipid) for four hours at room temperature. To optimize the conjugation, this mixture was diluted to 2 mM phospholipid concentration with 25 mM HEPES, 25 mM MES, 150 mM NaCl, pH 6.7 buffer. Uncoupled thiolated mAb was separated from liposomes conjugated to mAb by passing through a Sepharose-CL-4B (Sigma) column (1x25 cm) eluting with 25 mM HEPES, 150 mM NaCl, pH 7.4 buffer. (For reviewing MPB-PE coupling procedures: Martin and Papahadjopoulos, 1982; Heath, 1987 and Martin et al., 1990).

In the second method, because of the abundance of ß-galactose receptors on hepatocytes, we targeted liposomes to galactose receptors by means of three different ligands, lactosylceramide (LAC), asialo-GM_1 ($ASGM_1$), and asialofetuin (AF), recognized by the ß-galactose receptors. Lactosylceramide and asialo-GM_1 are glycolipids, which can be easily incorporated into liposomes by simply mixing them with HSPC and CH in a molar ratio of HSPC:CH:glycolipid 10:5:1. The procedures for forming these two kinds of glycolipid-incorporated liposomes were the same as those used to produce plain liposomes. The liposomes were extruded to approximately 100 nm in diameter. For asialofetuin, a glycoprotein, the biotin-avidin method was used to establish a specific conjugation between asialofetuin and liposomes (For a description of the biotin-avidin method see Loughery et al., 1987 and 1990).

Biotin-PE (0.02 mM stock solution in chloroform) was added during the formation of liposomes (HSPC:CH:Biotin-PE 10:5:0.01 moral ratio). The liposomes (HSPC:CH 2:1) incorporated with biotin-PE were formed by using the same procedures as for plain liposome formation. After incubation with avidin (2 mg/ml in 10 mM TES, 150 mM NaCl, pH 7.4 buffer) at a ratio of incorporated biotin-PE:avidin of 1 μmol:1 μmol (or 1 μmol:66 μg) at room temperature, liposomes containing biotin-PE bound avidin and formed the avidin-biotin-liposome complex. By passage through a Sepharose CL-4B column (1x20 cm) eluting with TES, pH 7.4 buffer, unbound avidin was separated from avidin-biotin-liposomes. The -NH_2 group of asialofetuin reacted with NHS-biotin by attacking the N-hydroxysuccinimidyl group, the leaving group of NHS-biotin, to yield biotinylated asialofetuin. After incubating asialofetuin (10 mg/ml in sodium borate, pH 8.8 buffer) with NHS-biotin (10 mg/ml in dimethyl sulfoxide, DMSO) at a ratio of 10 mg asialofetuin/1 mg NHS-biotin for four hours at room temperature, non-biotinylated asialofetuin was separated from biotinylated asialofetuin by passing through a Sephadex G-25 column (1x25 cm) using TES buffer, pH 7.4. The protein concentration of biotinylated asialofetuin was determined by using the BioRad™ reagent.

After incubation at room temperature with biotinylated asialofetuin (phospholipid:biotinylated asialofetuin 1:0.001 molar ratio) for 30 minutes, avidin-biotin-liposomes bound to biotinylated asialofetuin and formed asialofetuin-biotin-avidin-biotin-liposomes. Unbound biotinylated asialofetuin was separated from asialofetuin-biotin-avidin-biotin-liposomes by passage through a Sepharose CL-4B column (1x30 cm) with TES buffer, pH 7.4.

(4) Determination of Ligand Density on the Surface of Liposomes

The fluoraldehyde protein/peptide assay (developed by PIERCE, USA) was adopted to measure the density of mAb on the surface of liposomes. Orth-phthalaldehyde, the major component of assay reagent, reacts with a primary amine on a protein (or peptide) in the presence of 2-mercaptoethanol to form a fluorescent-labelled protein. Intensity of fluorescence can thus be converted into protein concentration. By comparison to a set of protein standards of appropriate concentration in the same diluent as samples, the density of mAb on the surface of the liposome was determined. Liposomes coupled to mAb were prepared as described above. The final concentration of phospholipid (in this case, HSPC) was measured by using the Bartlett method (Bartlett, 1959).

Five hundred μl of mAb-liposome suspension in PBS buffer (or plain PBS buffer for blank sample) and 100 μl of protein assay reagent were put into a quartz cuvette and incubated for 5 minutes at room temperature, with continuously stirring by a magnetic mini stir bar. The fluorescent labelled molecule has an excitation wavelength of 360 nm and fluoresces at 455 nm. The fluorescence intensity of sampled liposomes at 455 nm was measured. All fluorometric measurements were done on an Hitachi MPF-4 fluorescence spectrophotometer (Hitachi, Co., Ltd., Japan). These results were compared with a standard curve of free mAb in PBS buffer (10x, 20x and 40x dilutions of a standard solution, 100 μg mAb/ml PBS, pH 7.4), and the density of mAb conjugated was calculated from the ratio of fluorescent intensity at 455 nm of the standard to that of sampled liposomes and expressed as μg of mAb/μmol of phospholipid.

^{125}I-labelled asialofetuin (^{125}I-AF) was prepared by incubating asialofetuin and ^{125}I at a ratio of 500 μg:2 mCi of ^{125}I in 1000 μl of 100 mM Tris buffer, pH 7.0, with the presence of two beads of Iodobeads™. 2 mCi of ^{125}I was diluted to 500 μl in 100 mM Tris buffer, pH 7.0. After incubation of Iodobeads with ^{125}I for five minutes at room temperature, 500 μl of asialofetuin solution (1 μg AF/μl Tris buffer) were added into the reaction vial. After incubation for thirty minutes at room temperature, free ^{125}I was separated by passage through a disposable Sephadex G-25 column (PIERCE) with 10 mM TES buffered saline, pH 7.4. ^{125}I-labelled asialofetuin-containing fractions were pooled after measuring the absorbency at 280 nm and were stored at 4°C. Adequate amounts of ^{125}I-labelled asialofetuin were mixed with 2 ml of asialofetuin (10 mg/ml in 0.1 M sodium borate, pH 8.8 buffer). Biotinylated asialofetuin was made as described previously and the final concentration of biotinylated asialofetuin was determined by BioRad protein assay. The radioactivity of ^{125}I was measured by using a Beckman 8000 Gamma Counter (USA). The specific activity, cpm of ^{125}I/μg of biotinylated asialofetuin, was calculated. Liposomes containing biotin-PE were then prepared and incubated with ^{125}I-labelled biotinylated asialofetuin as described above. Phospholipid concentration was determined by Bartlett method (Bartlett, 1959).

By determining the final radioactivity of ^{125}I in the liposome samples and applying the specific activity of radioactivity to protein, the density of asialofetuin on the surface of liposome was calculated and expressed as μg of asialofetuin/μmol of phospholipid.

(C) *Uptake of Liposomes Containing Ligands Recognized by ß-Galactose Receptors and Competition Experiments*

Hepatoma cells (transfected or non-transfected) were cultured in six-well plates (Falcon) with an initial cell density of 2x10^5 cells per well, and grown to confluent monolayers. The purpose of waiting for confluence is to establish uniform experimental conditions in order to improve reproducibility of the results. When the plates were confluent, usually four to five days after seeding, one plate (six wells) of cultured cells was washed with 1 ml of phosphate buffered saline, pH 7.4 (PBS, Appendix I), three times. In each well, washed cells were trypsinized with 330 μl of trypsin (0.05% + 0.35 mM EDTA in Hank's buffer, GIBCO) at 37°C, in an incubator. After incubation for 7 to 10 min., the trypsinization was stopped by adding 1 ml of MEM medium into each well. Trypsinized cells were collected in a final volume of 1.33 ml for each well sample. Collected cell samples were transferred into centrifugation tubes and spun down at 500xg for 7 to 15 min. The supernatants were removed. In order to count the cell number of each well, these samples were further re-suspended with a suitable amount of PBS buffer. Every confluent well was found to have an average of 4×10^6 cells determined by either utilizing a haemocytometer (Reichert-Jung™, Cambridge Instrument INC., NY. USA) to count the cells, stained with 0.04% trypan-blue, under microscope, or a Coulter-Counter™ (Coulter Electronics INC., Hialeah, Florida) to count the cells re-suspended in PBS. This figure, 4×10^6 cells/well, was generally used to normalize the experiment data to cell number.

The experimental plates were incubated with different concentrations of various ^{3}H-labelled liposome suspensions (in PBS, pH 7.4) and triplicate samples were counted in every case. To cover the cells completely, 200 μl of liposome suspension was added into each well. Plates were shaken gently until added liposomes spread evenly in the wells and covered the cells completely. After incubation at 37°C, in 5% CO_2, for different time courses (1 hr., 2 hrs. and 4 hrs.), the treated hepatoma cells were washed with 1 ml of PBS three times to remove unattached liposomes. Then, the cells were trypsinized and harvested as mentioned above. Each sample (with a final volume of 1.33 ml) was collected in a 5 ml scintillation vial and 3.2 ml of scintillation fluid for ß-counting was added into each vial. Sample vials were vigorously vortexed before placing into the ß-counter. By determining the amount of bound and/or internalized radioactivity of [^{3}H]CHE added during the formation of liposomes, we were able to measure the liposome uptake by cultured cells. A Beckman scintillation counter (LS 6800 or 7500, Beckman Instrument INC., US) was used to measure the cpm of radioactivity.

To survey the specificity of uptake of different liposomes, a series of competition experiments were performed. In the case of mAb-targeted liposomes, 200 μl of free mAb against HBsAg (or free sheep IgG, Sigma) solution (0.6 mg/ml PBS, pH 7.4) was added to each well prior to the treatment of liposome suspensions. After incubation with free mAb (or free sheep IgG) at 37°C for 15 minutes, 200 μl of liposome suspension (1000 nmol PL/ml) was added into each well to make a final phospholipid concentration of 500 nmol PL/ml and the plates were kept in an incubator for another one hour. Treated cells were then washed, trypsinized, and harvested as mentioned before. Liposome uptake was determined by counting radioactivity associated with the hepatoma cells, as mentioned in the section on uptake experiments. The results were compared with data from control samples, which were previously incubated with 200 μl of plain PBS instead of mAb, or sheep IgG (a non-recognized antibody) in PBS.

The competition experiments for the ß-galactose receptor were performed by incubating free asialofetuin (50 nmol/ml PBS, pH 7.4) with cells at 37°C, 15 min. prior to the incubation of liposomes conjugated/incorporated with various ß-galactose receptor ligands. Procedures for these experiments were the same as those in the competition experiments for mAb-conjugated liposomes.

(D) *Entrapment of Antiviral Drug in Liposomes and Leakage Experiments*

Antiviral drug, ddGTP, was entrapped into asialofetuin-coupled liposomes (AF-liposomes) during the rehydration step of liposome preparation. 1 ml of ddGTP solution (10 mg/ml in 25 mM HEPES, 150 mM NaCl, pH 7.4 buffer) was used to rehydrate 20 μmoles of HSPC:CH:biotin-PE lipids at 2:1:0.002 molar ratio. Liposomes were made as described and free ddGTP was separated from liposomal ddGTP by passing the liposome suspension through a Sepharose-CL-4B column, eluting with 25 mM HEPES, 150 mM NaCl, pH 7.4 buffer. ddGTP was also encapsulated in plain liposomes (HSPC:CH 2:1) during the rehydration step. To mimic the experimental conditions for AF-liposomes, which were passed through Sepharose CL-4B columns two times during their formation (for separation of unbound biotinylated AF and of free ddGTP, respectively), the ligand-free (plain) liposomes loaded with ddGTP were again passed through a Sepharose CL-4B column after entrapment.

To study the entrapment efficiency/ratio of ddGTP and its leakage from plain or AF-liposomes, 2 μl of ^{32}P-dGTP was mixed with 1 ml of ddGTP solution (5 or 10 mg/ml HEPES buffer) and entrapped into ^{3}H-CHE-labelled plain or AF-liposomes, as mentioned above. After encapsulation, 1 ml of plain or AF-liposomes containing ddGTP were immediately passed through a 1x30 cm Sepharose CL-4B column eluted with 25 mM HEPES buffered saline, pH 7.4. Fifty eluted fractions (1 ml for each fraction) were collected. The radioactivity profiles of both ^{3}H and ^{32}P were obtained and dealt with as follows.

Because the radioactivity of ^{3}H and ^{32}P represented the presence of phospholipid (liposomes) and ddGTP, respectively, any leakage of ddGTP from liposomes would result in a lower ^{32}P peak, while the ^{3}H peak would remain the same. The accumulated radioactivity of ^{32}P for the pooled liposome peak was calculated and normalized by dividing by the accumulated ^{3}H radioactivity in liposome peak. Results obtained immediately after encapsulation was set as time zero (T_0). The same procedures as above were repeated for samples incubated at 37°C or 4°C for different periods of time. Through a comparison of results at different times and temperature, the leakage rates were calculated.

By tracing the radioactivity of ^{32}P-labelled dGTP added during rehydration, the ratio of entrapped ddGTP to phospholipid concentration of liposomes was measured and expressed as μg of ddGTP/μmol of phospholipid. The efficiency of entrapment (% of initial ddGTP ended up in liposomes) was also calculated. A Beckman 3000 scintillation counter was used to measure the radioactivity of ^{32}P.

(E) *Inhibition of HBV Replication, in Hepatoma Cells Transfected with HBV, by Liposomal Dideoxyguanosine Triphosphate (ddGTP)*

(1) Sterilization of Liposomes and Free ddGTP Solution

To prevent the contamination of hepatoma cell cultures by bacteria in the long term treatment protocol, liposomes and free ddGTP solutions, used for incubation with hepatoma cells, were sterilized. A set of syringe top extruders (Syringe Holder, Nucleopore™, USA) and several polycarbonate filters (0.2 μm) were autoclaved for 20 minutes in an AMSCO autoclave (American Sterilizer Co., Erie, Pennsylvania). Various liposome suspensions and free ddGTP solutions were sterilized by pushing them through the autoclaved filter and collecting in sterilized vials. These vials were then stored at 4°C for further experiments. All the liposome suspensions were used within 24 hours after preparation.

(2) Using Liposomal ddGTP to Inhibit HBV Replication in HBV-transfected Hepatoma Cells

2.2.15 cells were cultured in six-well plates and medium was changed every three days as described previously. When the wells became confluent, cells were treated with various sterilized liposomes (empty plain liposomes, empty AF-liposomes, plain liposomes containing ddGTP, and AF-liposomes containing ddGTP) or free ddGTP in PBS. All liposomes were at a concentration of 1 μmole phospholipid/ml in PBS. Medium was first removed from each well. Three hundred μl of a liposome suspension (or free ddGTP 250 μg/ml PBS) was added to each well. After incubation at 37°C for one hour, the unattached liposomes were removed by washing with 1 ml of PBS three times. Two ml of fresh MEM medium was then added into each treated well. The same procedures were repeated every other day during a 14-day-protocol. On the fourteenth day after the first treatment, cells were washed with PBS-EDTA (0.2 g of EDTA/L PBS, pH 7) and digested by incubating with 300 μl of pronase E (Sigma) solution (2 mg/ml PBS) for each well, at 37°C overnight.

(3) DNA Extraction from HBV-transfected Hepatoma Cells, Nick Translation and Dot Blotting

The digested cells of each well were collected in a 5 ml test tube and 1.3 ml of purified phenol was added into each tube to extract HBV-incorporated hepatoma DNA. After vigorous vortexing, these tubes were centrifugated for 20 minutes, at 2500 rpm (1200xg, Beckman model-TJ6). The DNA of hepatoma cells (incorporated with HBV-DNA) was then precipitated by incubating with ethanol (final concentration 50-75%, v/v, in each sample) in the presence of 2 M ammonium acetate (final concentration 0.2 M). After incubation overnight, samples were dried by evaporating the ethanol and re-dissolved into 1 M NaOH solution (200 μl for each sample). Boehringer

Mannheim Kits for nick translation were purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and the ^{32}P-HBV-DNA probe bound specifically to a segment of HBV-DNA sequence was freshly made as described in "Biochemical Analysis for Molecular Cloning" (Sambrook et al., 1989). In brief, the plasmid pAM 6 DNA which carries one copy of HBV cloned into the E.coli plasmid vector (Moriarty et al., 1981) was radiolabelled utilizing the nick translation method developed by Rigby et al. (1977), with modifications. A 20 μl reaction mixture containing 1x nick translation buffer, 400 pmoles each of dGTP, dATP and dTTP, 220 ng pAM 6 DNA, 50 μCi [α-^{32}P]dCTP (specific activity = 3000 Ci/mmole), and 2 μl of the enzyme mixture (DNA polymerase I + DNase I) provided with the Nick Translation Kit was incubated at 15°C for one hour. Stop solution at 37°C (20 μl of 0.1M EDTA, 1% SDS, 100 μg/ml herring sperm DNA, pH 7.5) was added to terminate the reaction, followed by heating at 65°C for 5 min. Specific incorporation of radiolabelled deoxynucleotide into the DNA was routinely estimated using a trichloroacetic acid (TCA) precipitation assay (Sambrook et al., 1989). The radio-labelled DNA probe was stored at -70°C and was utilized within 1 week of preparation.

HBV-DNA extraction samples (20 μl for each sample) were spotted and immobilized on a piece of water-soaked hybridization filter paper (Hybond™, Amersham) in a microfiltration apparatus (Bio-Dot; BioRad Laboratories). Blotted Hybond paper was left on a piece of filter paper soaked with 100 ml of neutralization solution (Appendix I) for 20 min. After neutralization with 2 successive applications of 100 ml of neutralization solution, Hybond paper was dried and wrapped in a piece of plastic wrapping membrane (Saran Wrap™). Wrapped Hybond paper was exposed to ultraviolet (UV) irradiation for 3 min. on a UV Transilluminator (UVP, Inc.; 254 nm; total exposure = 1.44 J/cm^2). The hybridization procedure outlined by Sambrook and coworkers (1989) was followed with modifications. The Hybond paper was transferred into a heat-sealable plastic bag. The bag was filled with 10 ml of prehybridization solution (42°C; freshly prepared, Appendix I) and air bubbles were completely exiled before sealing the bag. The bag was left in a water-bath at 42°C for 3 hours.

After pre-hybridization, a corner of the bag was cut off and sufficient amount of ^{32}P-probe, denatured by heating at 96°C for 2 min., was added into the bag to make a final solution containing 1 x 10^6 cpm/ml, approximately. The bag was resealed after expulsion of the air and gently mixed and submerged in the 42°C water bath. After incubation for 24 hours, ^{32}P-probe was then bound to the HBV-DNA fragments. Removal of non-specifically bound probe from the filter was accomplished by a brief rinse of the filter with 100 ml of 1x SSC/0.1% SDS [40 ml of 20x SSC (Appendix I), 8 ml of 10% SDS, and 752 ml of double distilled water] followed by 2 successive 45 min. washes with 200 ml of the same solution at room temperature with gentle, constant agitation. Any remaining probe that was partially bound to the immobilized DNA was removed by washing the filter twice for 45 min, at 65°C in 500 ml changes of 1x SSC/0.1% SDS. The damp filter was protected by Saran Wrap™. In the dark room, a piece of Kodak X-ray film (Kodak X-OMAT AR) was put on the hybridization paper blotted with DNA samples. The film was then sandwiched between the immobilized filter and an intensifying screen in a film chart, sealed with aluminum foil, and kept in -70°C freezer for 3 to 5 days. The exposed film was then developed by using X-ray film processor (Scan-1™, Kodak, Canada). The intensity of ^{32}P radioactivity is proportional to the amount of binding between probe and HBV-DNA. Thus, the developed image reflects the intensity of ^{32}P radioactivity and the inhibition of HBV-DNA replication by liposomal ddGTP.

(F) *Internalization of Liposomes Containing Fluorescent Probe by Hepatoma Cells Transfected with HBV*

The interaction of liposomes with 2.2.15 cells was monitored with the fluorescent compound HPTS (1-hydroxypyrene-3,6,8-trisulfonic acid) which could be entrapped in the liposomes. HPTS entrapped in plain liposomes (or AF-liposomes) were prepared as described by Daleke and coworkers (1990). 2×10^5 cells were cultured in Falcon tissue culture dishes (30 mm radius) and grown to confluence as monolayers. Five hundred μl of HPTS-liposomes (1 μM phospholipid in PBS supplemented with Ca^{2+}, Mg^{2+}, and glucose) was added into the dishes. After incubation for two or twenty-four hours at 37°C, treated cells were washed with 5 ml of PBS three times to remove unattached liposomes. Cells were then trypsinized, collected, and resuspended into a concentration of 2×10^5 cells/ml PBS, for fluorometric measurements (on an Hitachi MPF-4 fluorescence spectrophotometer). The pH-dependent property of HPTS was utilized to monitor the incorporation of fluorescent liposomes into cells and post-delivery pH changes associated with the uptake into cell compartments with different pHs. The ratio of the fluorescent emission at 403nm and 450nm (slit width, 4.5nm) to the emission after excitation at 413nm (slit width 4.5nm) were calculated from the peak heights (in centimeter) and used to estimate the pH of the environment to where the probe was exposed.

(G) *Intrahepatic Distribution of Liposomes Containing Asialofetuin and the Effect of the Presence of Polyethylene Glycol in Liposomes*

PEG-DSPE was added into the phospholipid mixture (labelled with [^{3}H]-cholesterol oleylether, [^{3}H]-COE, Amersham) and liposomes were formed as described previously. To allow passage through liver sinusoids (100-150 nm in diameter; Meijer et al., 1992), the initial diameter of liposomes was $\leq$ 85 nm. Asialofetuin was conjugated on the surface of liposomes (with or without PEG-DSPE) by using the avidin-biotin method mentioned before. In this case, conjugation ratios were determined by Lowry assay (Lowry et al., 1951), after precipitation of liposomes with trichloroacetate (final concentration: 36% in each sample) and centrifugation at 500xg for 5 min. Four different compositions of liposomes, i.e., plain liposomes (HSPC:CH, 2:1 molar ratio), plain liposomes containing PEG (PEG-liposomes, HSPC:CH:PEG-DSPE 2:1:0.1 molar ratio), AF-liposomes (AF-HSPC:CH,2:1 molar ratio), and AF-liposomes containing PEG (AF-PEG-liposomes, AF-HSPC:CH:Biotin-PE:PEG-DSPE 2:1:0.002:0.1 molar ratio), were injected into male Wag/Rij rats (TNO, Rijswijk, The Netherlands), weighing 220-260 gram, by the intravenous route (penis vein). Rats received 3 μmol of liposomal phospholipid in less than 1 ml of 25 mM HEPES saline (pH 7.4). Blood samples were taken from the tail vein. After incubation on ice for two hours, serum was separated from the whole blood by centrifugation at 500xg for 5 min. By reading the radioactivity of serum samples, the blood clearance curve and the half-life in plasma for each type of liposome was determined.

At 24 hours after injection, the animal was anaesthetized with diethylether and sacrificed. The spleen was removed and homogenized, and the liver was pre-perfused at 37°C for 10 min. *in situ* with Hank's buffer (pH 7.35, Appendix I) to remove blood. Before perfusion with collagenase, one piece of liver was taken, homogenized and total uptake of liposomes by liver was calculated from measured radioactivity in the sampled liver homogenate, by using equation below:

Total Liver Uptake = cpm of counted sample x *volume factor x ([total weight of liver after perfusion] ÷ [weight of a piece of sampled liver])

*volume factor = (total volume of sampled liver homogenate) ÷ (volume of counted liver homogenate)

Total uptake of liposomes by spleen was obtained from measuring radioactivity in the total spleen homogenate, by the equation below:

Total Spleen Uptake = cpm of counted sample x *volume factor

* volume factor = (total volume of spleen homogenate) ÷ (volume of counted spleen homogenate)

For hepatocyte and Kupffer cell isolation the liver was perfused at 37^0C for 30 min. with collagenase (0.04% in Hank's buffer/1 mM $CaCl_2$, pH 7.35) (Roerdink et al., 1981 and Scherphof et al., 1983). The perfused liver was removed and washed and hepatic cells (i.e., parenchymal, Kupffer, endothelial, fat-deposit cells, etc.) were collected in 0.3% bovine serum albumin (in Hank's buffer, pH 7.35; on ice). Collected cells were further handled at 4^0C as described by Nagelkerke et. al.(1982 and 1983) to isolate the parenchymal, Kupffer and other cell fractions. The protein concentration of each cell fraction was determined by Lowry assay (Lowry et al., 1951). The association of liposomes with a specific type of cells was monitored by tracking the ^{3}H radioactivity read in each respective cell fraction. Specific activity of liposomal association for each cell fraction was calculated and expressed as dpm/mg of protein. The contribution of parenchymal, Kupffer, and endothelium cells to total liver proteins is 92.5%, 2.5%, and 3.3%, respectively (Blouin et. al., 1977 and Meijer and Ziegler, 1993). According to these figures and specific activity of liposomal association with each cell type, the distribution of liposomes in liver was estimated.

III. RESULTS

(A) *Physical Characterisation of Liposomes*

(1) Liposome Size

The detailed compositions of various liposomes discussed in this study are shown in Table 1. The average diameter of different liposomes was determined as described before. The liposomes are all within an initial diameter of 100 ± 10 nm. After the coupling procedure, the AF-liposomes and mAb-liposomes have larger diameters than LAC-, ASGM-, or plain liposomes. The difference in size is not taken as a significant factor in the *in vitro* investigations, since the size of AF-liposomes or mAb-liposomes is close to that of the other liposomes (Table 2).

(2) Density of Ligands on the Liposomal Surface

The density of mAb on liposomal surfaces was determined by comparing the fluorescent intensity of mAb-liposomes to a standard curve of free mAb. By using methods previously described, the density of mAb on the liposomal surface was calculated to be 13.3 ± 0.5 μg mAb/μmol PL.

^{125}I-Asialofetuin was made and utilized to quantitate the asialofetuin conjugation ratio. It is found that every 1 μmole of phospholipids can conjugate approximately 26 μg (i.e., 5×10^{-4} μmol) of asialofetuin, by using the biotin-avidin conjugation technique. (Table 3A).

(3) Entrapment Ratio and Encapsulation Efficiency of ddGTP by AF-liposomes

^{32}P-dGTP was mixed with ddGTP to evaluate the efficiency of entrapment, as mentioned previously. Entrapped efficiency varied with experimental conditions (Table 3B). By using 5 mg of ddGTP in 1 ml HEPES buffered saline to rehydrate 5 μmoles of HSPC, every one μmole of phospholipids in liposomes can entrap 7.6 μg (i.e., 0.015 μmoles) of ddGTP. This means the efficiency of entrapment is less than 1%. When the initial concentration of HSPC was increased to 100 μmoles and the concentration of ddGTP remained at 5 mg/ml, the efficiency increased to 23%, i.e., about 11.7 μg of ddGTP/μmol PL.

Table 1 Composition and ligand density of various liposomes.

Composition is shown in molar ratio. The surface ligand density of AF-liposomes is based on the data of conjugation ratio. Because asialofetuin has three terminal galactose residues, the surface galactose density is three times of the conjugation ratio. Because lactosylceramide and asialo-GM_1 were incorporated into the lipid bilayers in a symmetrical manner, only approximately 50% of the lactosylceramide or asialo-GM_1 would be on the outer surface of the liposomes.

Composition

liposome type	composition	molar ratio
Plain liposomes	HSPC:CH	2:1
Plain PEG-liposomes	HSPC:CH:PEG-DSPE	2:1:0.1
AF-liposomes	HSPC:CH:Biotin-PE	2:1:0.002
AF-PEG-liposomes	HSPC:CH:Biotin-PE:PEG-DSPE	2:1:0.002:0.1
mAb-liposomes	HSPC:CH:MPB-PE	2:1:0.02
LAC-liposomes	HSPC:CH:LAC	2:1:0.2
$ASGM_1$-liposomes	HSPC:CH:$ASGM_1$	2:1:0.2
LAC-PEG-liposomes*	egg PC:CH:LAC:PEG-DSPE	2:1:0.2:0.1

Ligand density

liposome type	conjugation ratio (μmol ligand/μmol PL)	surface density (μmol galactose/μmol PL)
LAC-liposomes	0.1	0.05
$ASGM_1$-liposomes	0.1	0.05
AF-liposomes	$5.2x10^{-4}$	$1.56x10^{-3}$

* Scherphof et al., 1994. (egg PC : egg phosphatidylcholine)

Table 2 Average liposome diameter.

Liposome diameters (mean ± S.D., n = 3) were determined by measuring their dynamic light scattering on a Brookhaven BI-90 particle sizer. LAC, lactosylceramide; $ASGM_1$, asialo-monosialoganglioside, GM_1; AF, asialofetuin; mAb, mAb against HBsAg.

Liposome composition (molar ratio)	Initial diameter (nm)	Diameter after conjugation (nm)
HSPC:CH (2:1)	98 ± 4	ND
LAC-HSPC:CH (2:1)	103 ± 3	ND
$ASGM_1$-HSPC:CH (2:1)	102 ± 7	ND
AF-HSPC:CH (2:1)	101 ± 4	128 ± 6
mAb-HSPC:CH (2:1)	100 ± 3	139 ± 4

ND: not determined

Table 3A Conjugation ratio and diameter of AF-liposomes.

Conjugation ratio was determined by measuring the amount of ^{125}I-labelled asialofetuin conjugated on liposomes. AF-liposomes were composed of AF-HSPC:CH, 2:1 molar ratio. Mean ± S.D. (n=3).

Diameter and conjugation ratio of AF-liposomes	
Initial diameter	101 ± 4 nm
Diameter after conjugation	128 ± 6 nm
Conjugation ratio	26 ± 2 μg of AF/μmol PL

Table 3B Relationship between experimental conditions and entrapment ratio/efficiency.

AF-liposomes were loaded with ddGTP labelled with ^{32}P-dGTP at various initial phospholipid (PL) concentrations and ddGTP concentrations. The entrapment ratio/efficiency were determined by measuring the ^{32}P radioactivity remaining in the liposomes. Mean ± S.D., (n=3).

initial PL concentration	initial ddGTP concentration	entrapment ratio μg ddGTP/μmol PL	entrapment efficiency % of initial ddGTP
5 μmol/ml	5 mg/ml	7.6 ± 0.2	0.8 ± 0.02
100 μmol/ml	5 mg/ml	11.7 ± 0.9	23.4 ± 1.9
20 μmol/ml	10 mg/ml	14.9 ± 0.2	3.0 ± 0.04

The initial concentration of phospholipid and ddGTP used to prepare the liposomes (both AF-liposomes and plain liposomes) containing ddGTP for inhibition and leakage experiments was 20 μmol/ml and 10 mg/ml in HEPES buffered saline, respectively. Under these conditions, the ddGTP entrapment ratio was 15 μg ddGTP/μmol PL and encapsulation efficiency was 3%, approximately (Table 3B).

(4) Leakage of ddGTP from Liposomes

Liposomes (both plain liposomes and AF-liposomes) with entrapped ddGTP were very stable after preparation, during storage at 4°C or at 37°C. Table 4A,B shows the results for ddGTP leakage obtained at different temperatures for AF-liposomes (4A) and plain-liposomes (4B). For both AF- and plain liposomes, leakage of ddGTP was not detectable after storage at 4°C for up to 5 days. After incubation at 37°C for 24 hours, approximately 90% of ddGTP remained in either plain or AF-liposomes. After incubation at 37°C for 48 hours, approximately 80% of ddGTP remained in both compositions of liposomes. The half life for leakage of ddGTP from plain or AF-liposomes at 37°C was calculated from the percentage of ddGTP remaining in the liposomes and was found to be approximately 4 days and 6 days, respectively.

(B) *Uptake Experiments*

A series of experiments was performed to investigate the uptake of liposomes by hepatoma cells. Liposome uptake was examined as a function of various ligands recognized by transfected or non-transfected hepatoma cells. Here, liposomal uptake by cells was taken as indicating the total amount of liposomes associating with cells by any possible means, such as internalization, specific binding or non-specific adhesion.

(1) Plain Liposomes (Ligand-free Liposomes)

The uptake of plain liposomes (HSPC:CH, 2:1) by HepG2 and 2.2.15 cells is shown as Figure 1. It was found that the uptake by either cell line increased proportionally to the increase in phospholipid concentration and the difference

Table 4 Leakage of ddGTP from various liposomes.

ddGTP, labelled with ^{32}P-dGTP, was encapsulated in ^{3}H-labelled AF- or plain liposomes and leakage experiments were performed as described in Materials and Methods. AF- or plain liposomes were incubated at 4° or 37°C for different time periods. After incubation, 1 ml of liposome suspension was passed through a Sepharose CL-4B column and eluted with HEPES buffered saline, pH 7.4. Fifty fractions were collected. The accumulated radioactivity of ^{32}P and ^{3}H (from fraction 10 to 20) was calculated and found to be agreement with the amount of radioactivity added to the column in the liposome sample. The total radioactivity of ^{32}P associated with ddGTP-containing fractions was normalized by dividing by the total ^{3}H from the liposome fractions. The percentage of ddGTP remaining in liposomes was calculated by dividing the normalized radioactivity (^{32}P/^{3}H) obtained at different conditions by that obtained right after encapsulation (T_0). **A.** AF-liposomes (AF-HSPC:CH, 2:1); **B.** plain liposomes (HSPC:CH, 2:1).

4A. AF-liposomes

Temperature (°C)	Time (t) (hours)	Radioactive counts			% of ddGTP remaining in AF-liposomes at time t
		^{32}P (cpm)	^{3}H (cpm)	$^{32}P/^{3}H$	
no incubation	0	7904	123698	0.065	100%
4	48	8852	135175	0.066	101%
4	120	8433	138739	0.061	94%
37	2	9098	147986	0.061	94%
37	24	8150	137884	0.059	91%
37	48	7188	132440	0.054	83%

$T_{1/2}$ of ddGTP leaking from AF-liposomes in buffer at 37°C is approximate 6 days

4B. Plain liposomes

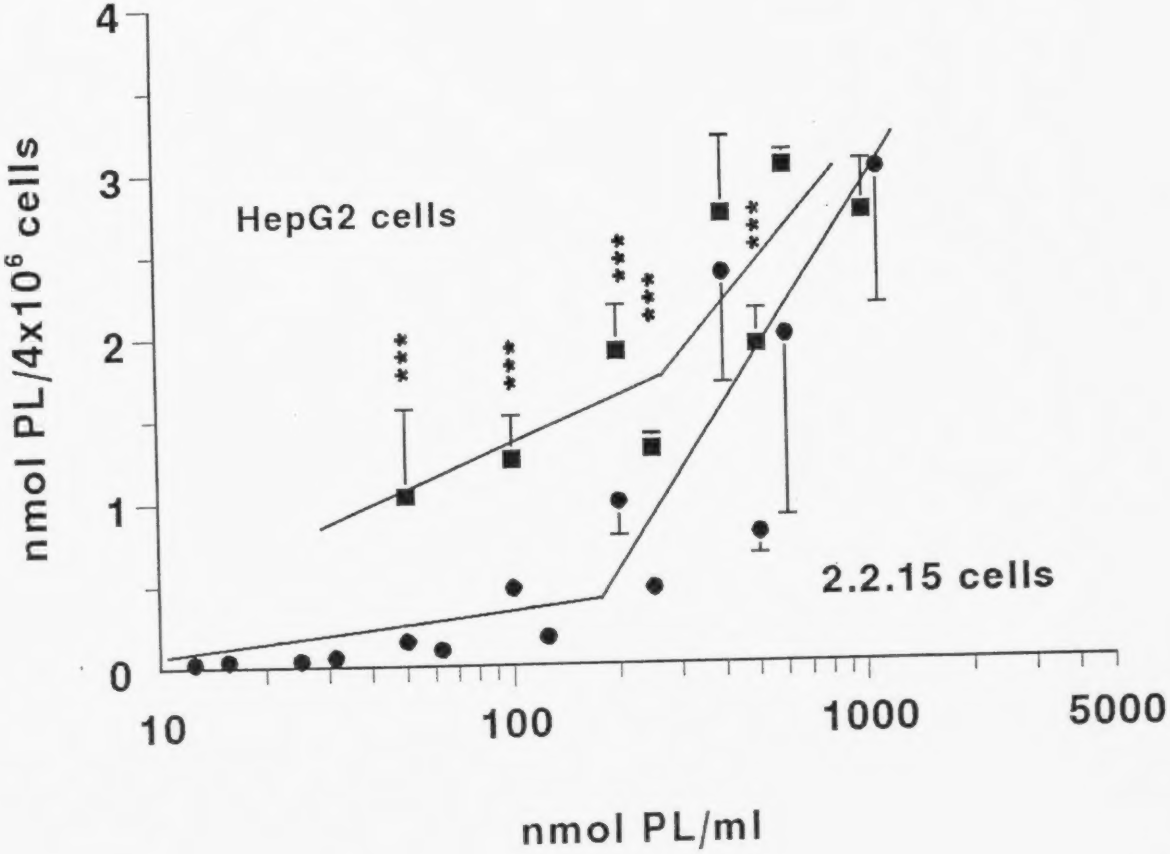
Temperature (°C)	Time (t) (hours)	Radioactive counts			% of ddGTP remaining in Plain liposomes at time t
		^{32}P (cpm)	^{3}H (cpm)	$^{32}P/^{3}H$	
no incubation	0	28533	253453	0.113	100%
4	24	25516	233204	0.109	96%
4	48	28466	259656	0.110	97%
4	120	24292	241381	0.101	89%
37	2	27205	256950	0.106	94%
37	24	26293	266555	0.099	88%
37	48	22560	251314	0.090	80%

$T_{1/2}$ of ddGTP leaking from liposomes in buffer at 37°C is approximate 4 days

Figure 1 Uptake of ligand-free liposomes (HSPC:CH, 2:1 molar ratio) by 2.2.15 cells and HepG2 cells.

Liposome suspensions of various phospholipid (PL) concentrations were incubated with HepG2 or 2.2.15 cells at 37°C for one hour and then washed, trypsinized and harvested as described in Materials and Methods. The liposomal uptake by different types of cells was calculated from the amount of ^{3}H-CHE-lipid label associating with the cells and was normalized to the uptake by 4×10^{6} cells. For the uptake by 2.2.15 cells (●), data were compiled from five separate experiments ($n = 3$ to 15). For the uptake by HepG2 cells (■), data were compiled from three separate experiments ($n = 3$ to 9). Significant level (determined by t-test) indicates the difference between the uptake by 2.2.15 and HepG2 cells. ✱✱✱ p value ≤ 0.001.

Uptake of Plain Liposomes by
2.2.15 and HepG2 cells



between the uptake of liposomes by HepG2 cells and by 2.2.15 cells was statistically significant, indicating that 2.2.15 cells associated with liposomes by a manner different from that of HepG2 cells.

(2) Lactosylceramide Liposomes

The uptake of liposomes containing lactosylceramide (LAC-liposomes) by 2.2.15 cells is shown in Figure 2 and 3. At low phospholipid concentrations (under 50 nmol PL/ml), the uptake of LAC-liposomes after one hour incubation showed no significant difference, as compared to the uptake of plain liposomes (Fig. 3). With increasing phospholipid concentration, the uptake of LAC-liposomes was increased over the uptake of plain liposomes. At high concentrations (over 500 nmol PL/ml), the LAC-liposomes show a two-fold-increased uptake by 2.2.15 cells, as compared to plain liposomes (Table 5).

The uptake of LAC-liposomes by HepG2 cells (Fig. 4) was higher than that seen for the 2.2.15 cell line. In general, at high phospholipid concentrations ($\geq$ 500 nmol/ml), uptake of LAC-liposomes by HepG2 cells after incubation was more than twice the uptake by the 2.2.15 cell line under identical conditions (Table 5; Fig. 2; Fig. 4). This suggests that the number of ß-galactose receptors on the 2.2.15 cell surface may be lower than that on the HepG2 cell surface, or the receptors on 2.2.15 cells are less able to recognize galactose residues on LAC-liposomes than are HepG2 cells.

(3) Asialo-GM$_1$ Liposomes

The uptake of liposomes containing asialo-GM$_1$ (ASGM$_1$-liposomes) was similar to the uptake of LAC-liposomes (Fig. 2; Fig. 4). Unlike LAC-liposome, at low phospholipid concentrations (under 50 PL nmol/ml), the uptake of ASGM$_1$-liposomes by 2.2.15 cells was significantly higher than the uptake of plain liposomes (Fig. 5). However, the overall uptake of ASGM$_1$-liposomes is not distinguishable from that of LAC-liposomes (Fig. 2, 4; Table 5).

For the HepG2 cell line, uptake of ASGM$_1$-liposomes was almost identical with

Figure 2 Uptake of targeted liposomes by 2.2.15 cells.

Liposome suspensions of various compositions (refer to Table 1) and phospholipid (PL) concentrations were incubated with 2.2.15 cells at 37°C for one hour. Cells were washed, trypsinized and harvested as described in Materials and Methods. Uptake was calculated from the amount of ^{3}H-CHE-lipid label associating with the cells. Data were compiled from 3 to 5 separate experiments. Lactosylceramide- (LAC-) liposomes (▲), n = 3-9; Asialo-GM$_1$- (ASGM$_1$-) liposomes (▼), n = 3-9; Asialofetuin- (AF-) liposomes (■), n = 3-15; Plain liposomes (●), n = 3-15.

Uptake of Targeted Liposomes by 2.2.15 Cells Compared with Plain Liposomes

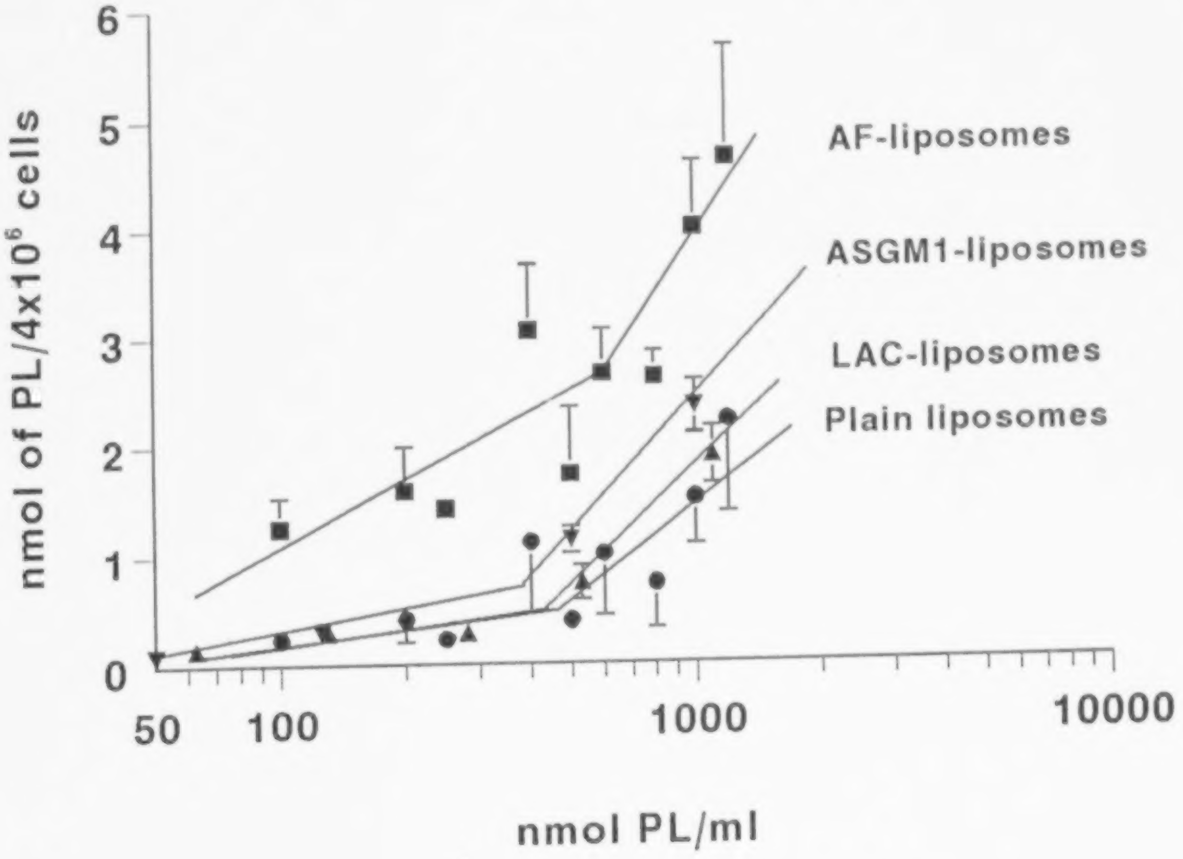


Figure 3 Comparison of Uptake, in 2.2.15 cells, of lactosylceramide-liposomes (LAC: HSPC:CH, 0.2:2:1 molar ratio) with ligand-free liposomes (HSPC:CH, 2:1 molar ratio).

Liposome suspensions were incubated with 2.2.15 cells at 37°C for one hour at various phospholipid (PL) concentrations and then washed, trypsinized and harvested as described in Materials and Methods. Liposomal uptake by 2.2.15 cells was calculated from the amount of ^{3}H-CHE-lipid label associating with the cells and normalized to the uptake by 4×10^6 cells. Data were from one experiment (n = 3). Plain liposomes (●); Lactosylceramide- (LAC-)liposomes (▲). Significant level (determined by t-test) indicates the difference between the uptake of plain and LAC-liposomes. ✱ p value ≤ 0.05.

Uptake of LAC-Liposomes by 2.2.15 Cells Compared with Plain Liposomes

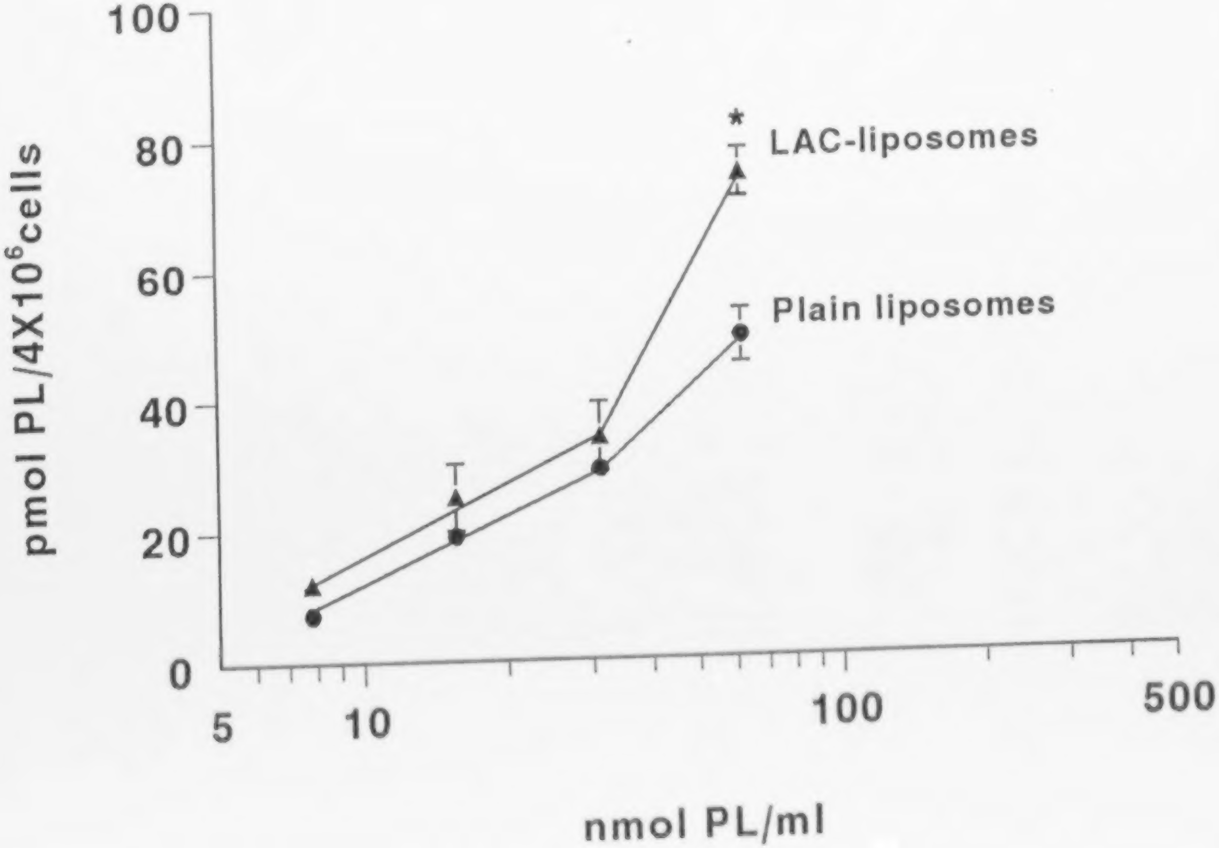


Figure 4 Uptake of targeted liposomes by HepG2 cells.

Liposome suspensions of various compositions (refer to Table 1) and phospholipid (PL) concentrations were incubated with HepG2 cells at 37°C for one hour. Cells were washed, trypsinized and harvested as described in Materials and Methods. Uptake was calculated from the amount of ^{3}H-CHE-lipid label associating with the cells. Data were compiled from 3 to 5 separate experiments. Lactosylceramide- (LAC-) liposomes (▲), n=3-9; Asialo-GM$_1$- (ASGM$_1$-) liposomes (▼), n=3-9; Asialofetuin- (AF-) liposomes (■), n=3-15; Plain liposomes (●), n=3-15.

Uptake of Various Liposomes
by HepG2 cells

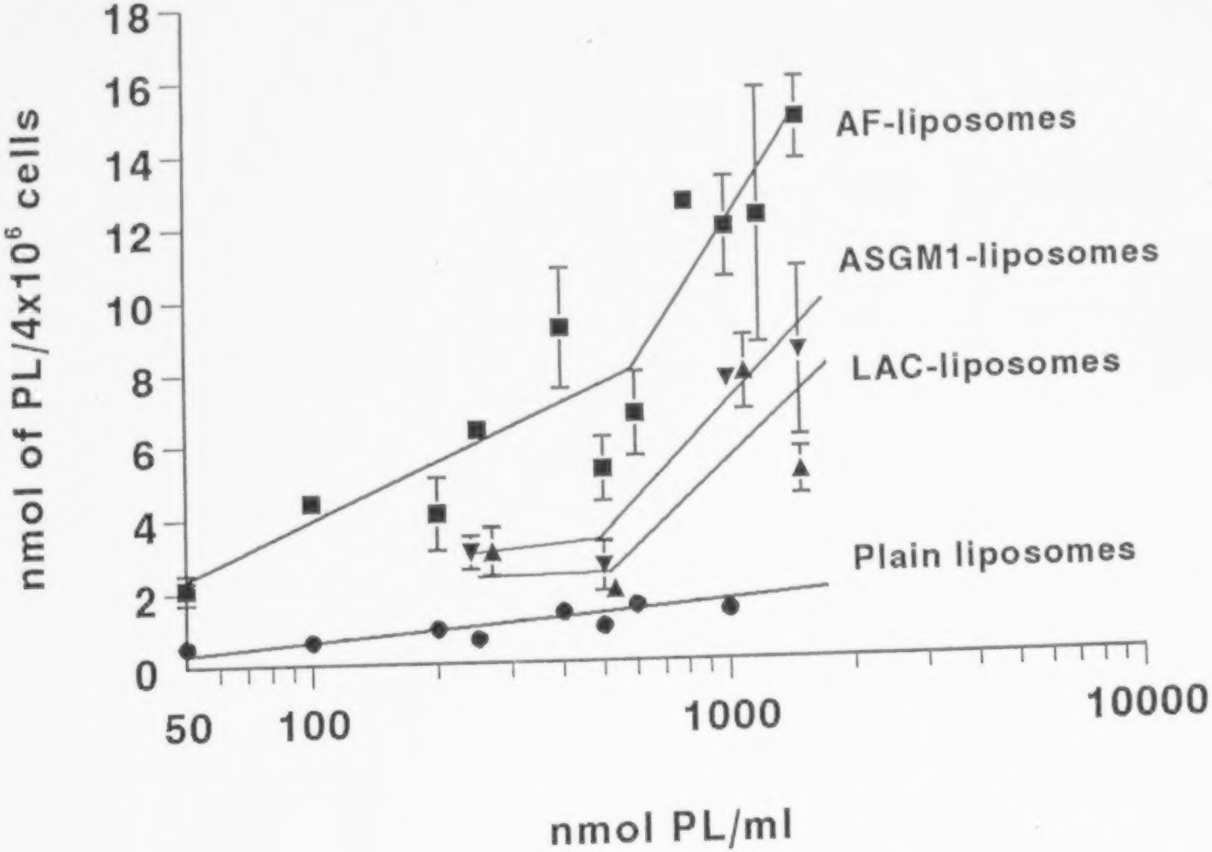


Table 5 Comparison of uptake of various liposomes by HepG2 or 2.2.15 cells.

Cells were incubated with various liposomes of different compositions at different phospholipid (PL) compositions at 37°C for one hour. Cells were then washed and harvested as described in the Materials and Methods section. Uptake (mean ± S.D., n=9) was determined from the amount of ^{3}H-CHE-lipid label associating with cells and normalized to the uptake by 4x10^6 cells.

Cell type	Liposome composition (molar ratio)	Uptake (nmol PL/4x10^6 cells)	
		500 nmol PL/ml	1000 nmol PL/ml
2.2.15 cells	AF-HSPC:CH (2:1)	1.6 ± 0.6	5.8 ± 1.1
	LAC-HSPC:CH (2:1)	1.2 ± 0.3	3.1 ± 0.7
	ASGM$_1$-HSPC:CH (2:1)	1.2 ± 0.1	3.7 ± 0.2
	mAb-HSPC:CH (2:1)	1.5 ± 0.3	3.1 ± 0.5
	HSPC:CH (2:1)	0.4 ± 0.06	1.5 ± 0.4
HepG2 cells	AF-HSPC:CH (2:1)	5.2 ± 0.9	12.0 ± 0.8
	LAC-HSPC:CH (2:1)	2.0 ± 0.3	7.8 ± 1.1
	ASGM$_1$-HSPC:CH (2:1)	2.6 ± 0.7	7.7 ± 1.3
	mAb-HSPC:CH (2:1)	1.0 ± 0.2	2.1 ± 0.7
	HSPC:CH (2:1)	1.0 ± 0.1	1.4 ± 0.2

the uptake of LAC-liposomes and was two-fold higher than the uptake of ASGM$_1$-liposomes by 2.2.15 cells (Fig. 2; Fig. 4; Table 5). This further suggests that the expression of ß-galactose receptors on HepG2 cells is different from that on the 2.2.15 cells.

(4) Asialofetuin Liposomes

Figure 6 shows the uptake of liposomes conjugated to asialofetuin (AF-liposomes) by 2.2.15 cells. It is clear that, even at a very low concentration (10 nmol PL/ml) of phospholipids, the AF-liposomes display a significantly higher uptake than plain liposomes (Fig. 7A). Similar observation is also seen in HepG2 cells (Fig. 7A). With increasing phospholipid concentration, the uptake of AF-liposomes increases proportionately. At 1000 nmol PL/ml, the uptake of AF-liposomes by 2.2.15 cells is more than three times the uptake of plain liposomes (Table 5, Fig. 2). Under the same conditions, LAC-liposomes or ASGM$_1$-liposomes show a two-fold increase in uptake by transfected hepatoma cells over plain liposomes.

In the case of HepG2 cells, the effect of increasing liposome uptake by the incorporation of asialofetuin is more obvious (Table 5, Fig. 7B). After incubating AF-liposomes (1000 nmol PL/ml) with HepG2 cells for one hour, the AF-liposome uptake reached 12 nmol PL/4x10^6 cells while the uptake of plain liposomes was under 2 nmol PL/4x10^6 cells. However, at 1000 nmol PL/ml, the uptake of AF-liposomes by 2.2.15 cells was 6 nmol PL/4x10^6 cells, only half of the uptake by HepG2 cells under identical conditions (Fig. 7B).

Incubating free asialofetuin with 2.2.15 cells prior to the treatment of various ligand-liposomes results in a 50% decreased uptake in every case (Fig. 8), indicating the uptake of ligand-liposomes is via the same receptors as those taking up asialofetuin, likely the ß-galactose receptors. For the 2.2.15 cell line, the uptake of ligand-liposomes was in the order of AF- > ASGM$_1$- ≥ LAC- > plain liposomes. This suggests that the uptake of liposomes by transfected hepatoma cells can be increased significantly by targeting to ß-galactose-receptors. Improved liposome uptake, via ß-

Figure 5 **Comparison of uptake, in 2.2.15 cells, of asialo-GM_1-liposomes ($ASGM_1$:HSPC:CH, 0.2:2:1 molar ratio) with ligand-free liposomes (HSPC:CH, 2:1 molar ratio).**

Liposome suspensions were incubated with 2.2.15 cells at 37°C for one hour at various phospholipids (PL) concentrations and then washed, trypsinized and harvested as described in Materials and Methods. Liposomal uptake by 2.2.15 cells was calculated from the amount of ^{3}H-CHE-lipid label associating with the cells and normalized to the uptake by 4×10^6 cells. Data were from one experiment ($n=3$). Plain liposomes (●); Asialo-GM_1- ($ASGM_1$-) liposomes (▼). Significant level (determined by t-test) indicates the difference between the uptake of plain and $ASGM_1$-liposomes. ** p value$\leq$0.01; *** p value$\leq$0.001.

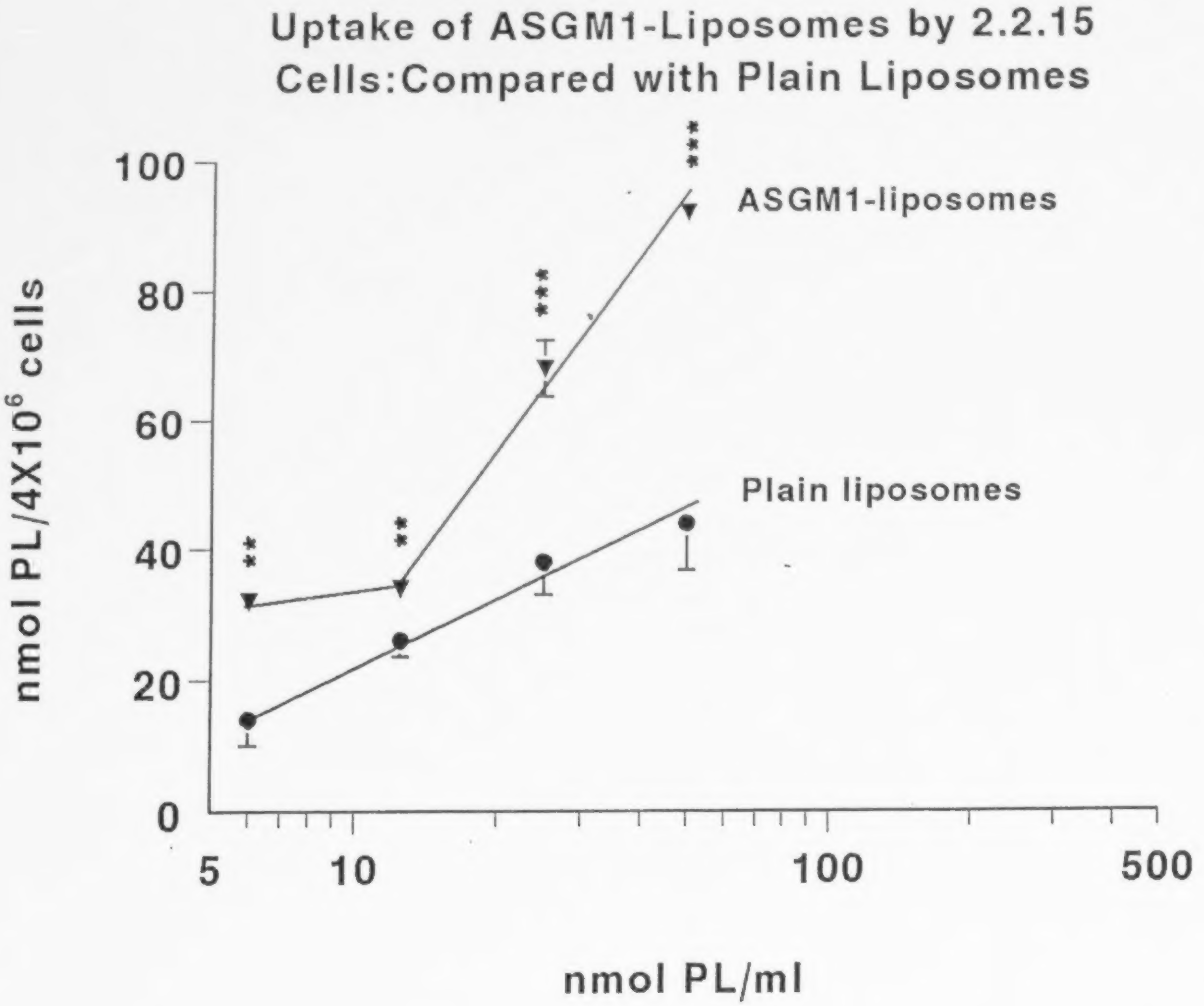

Uptake of ASGM1-Liposomes by 2.2.15 Cells:Compared with Plain Liposomes

Figure 6 Comparison of uptake, in 2.2.15 cells, of asialofetuin-liposomes (HSPC:CH:biotin-PE 2:1:0.002 molar ratio) with ligand-free liposomes (HSPC:CH 2:1 molar ratio).

Liposome suspensions of various phospholipid (PL) concentrations were incubated with 2.2.15 cells at 37°C for one hour and then washed, trypsinized and harvested as described in Materials and Methods. The data for liposomal uptake by 2.2.15 cells were calculated from the levels of radioactivity of ^{3}H-CHE associating with cells and normalized to the uptake by 4×10^6 cells. For the uptake of asialofetuin-(AF-) liposomes (■), data were compiled from five separate experiments (n = 3 to 15). For the uptake of plain liposomes (●), data were also from five separate experiments (n = 3 to 15). Significant level (determined by t-test) indicates the difference between the uptake of plain and AF-liposomes. ******* p value$\leq$0.001.

Uptake of AF-liposomes by 2.2.15 Cells Compared with Plain Liposomes

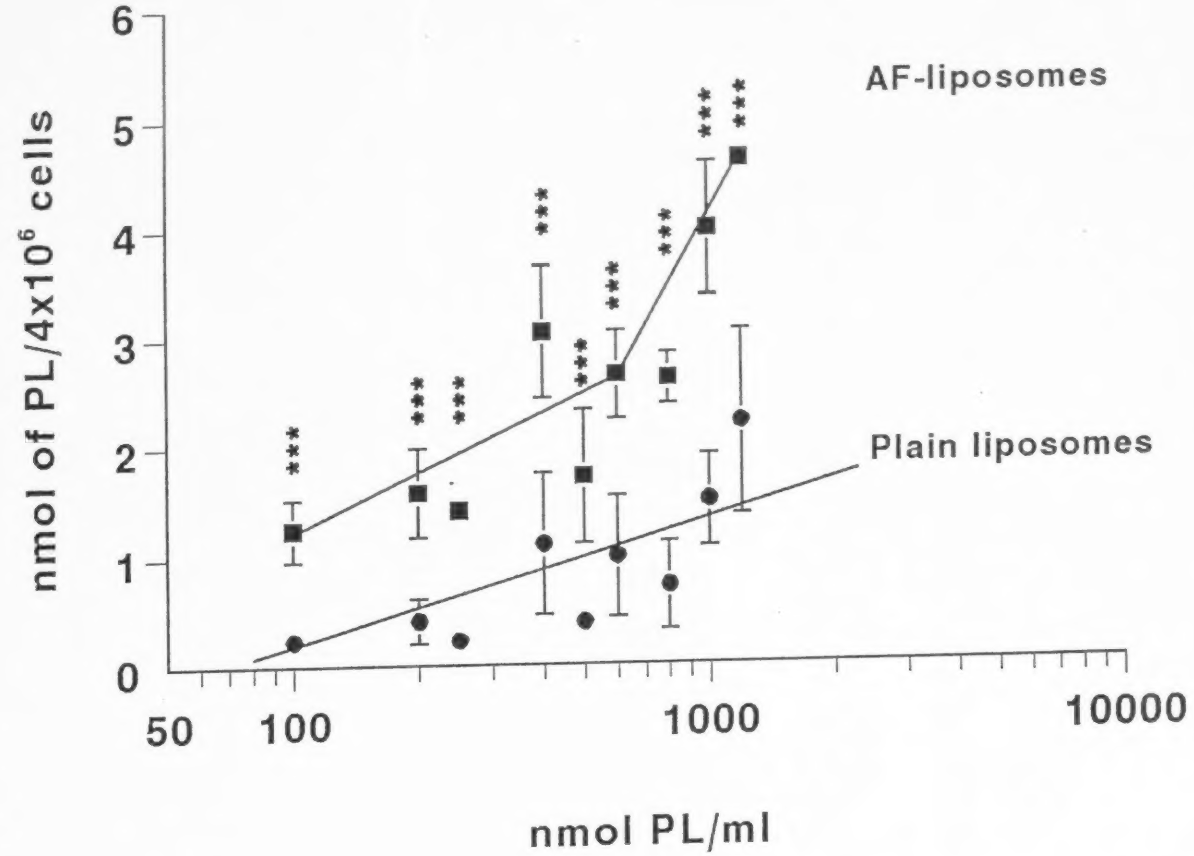
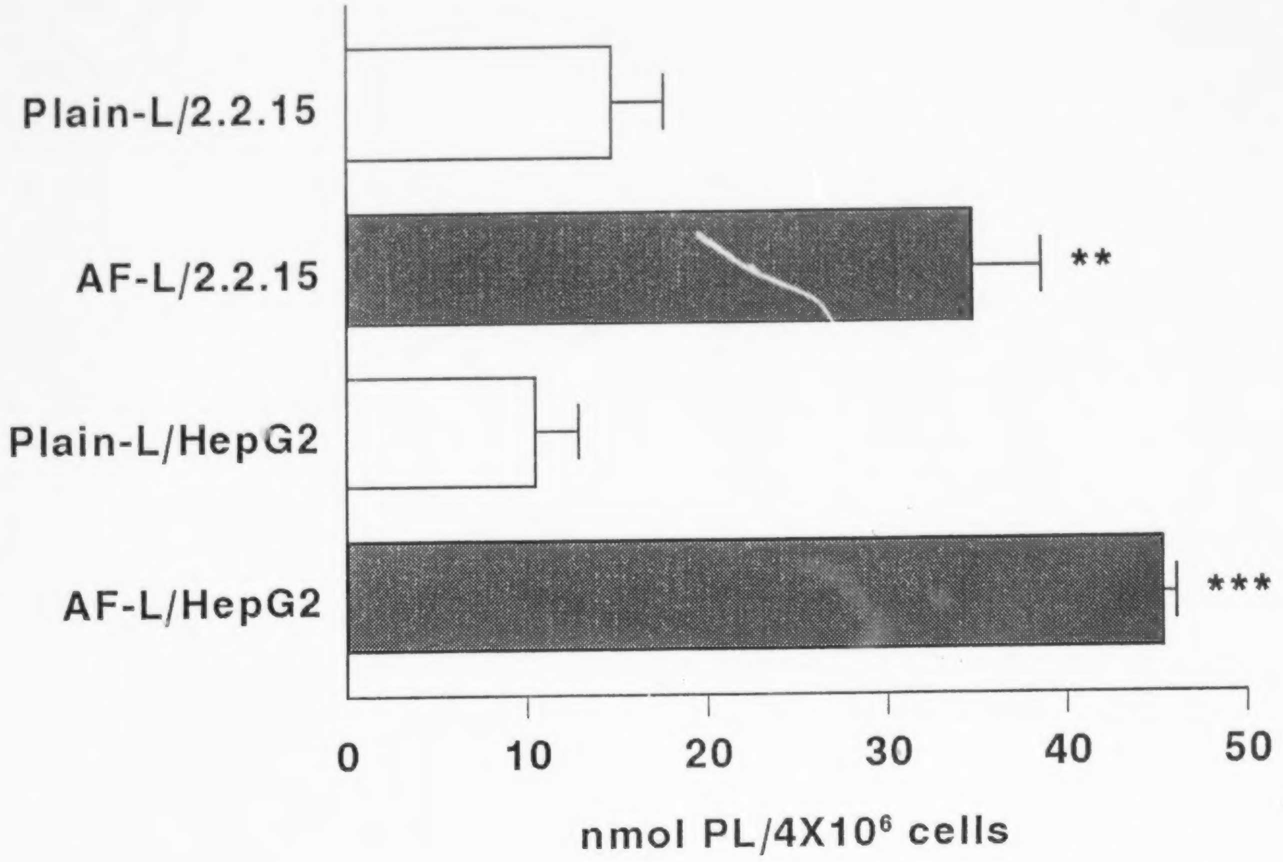


Figure 7A Comparison of uptake, in 2.2.15 or HepG2 cells, of asialofetuin-liposomes (HSPC:CH:biotin-PE 2:1:0.002 molar ratio) with ligand-free liposomes (HSPC:CH 2:1 molar ratio).

Liposome suspensions of 10 nmol phospholipid (PL)/ml were incubated with 2.2.15 or HepG2 cells at 37°C for one hour and then washed, trypsinized and harvested as described in Materials and Methods. The data for liposomal uptake by cells was calculated from the radioactivity of ^{3}H-CHE associating with cells and normalized to the uptake by 4x10^6 cells. Data were from two separate experiments (n = 3). Asialofetuin- (AF-) liposomes, shaded bar; Plain liposomes, empty bar. Significant level (determined by t-test) indicates the difference between the uptake of plain and AF-liposomes by each cell line. ✱✱ p value≤0.01; ✱✱✱ p value≤0.001.

Figure 7B Comparison of uptake of asialofetuin-liposomes (HSPC:CH:biotin-PE 2:1:0.002 molar ratio) by HepG2 cells with that by 2.2.15 cells.

AF-liposomes of various phospholipid concentrations were incubated with 2.2.15 or HepG2 cells at 37°C for one hour and then washed, trypsinized and harvested as described in Materials and Methods. The data for liposomal uptake by cells was calculated from the radioactivity of ^{3}H-CHE associating with cells and normalized to the uptake by 4x10^6 cells. Data were from five separate experiments (n = 3-15). (■) 2.2.15 cells; (▼) HepG2 cells. Significant level (determined by t-test) indicates the difference between the uptake of AF-liposomes by 2.2.15 and HepG2 cells. ✱✱✱ p value≤0.001.



A. Uptake of AF-Liposomes by 2.2.15/HepG2 Cells Compared with Plain Liposomes

B. Uptake of AF-liposomes by 2.2.15 Cells Compared with that by HepG2 Cells

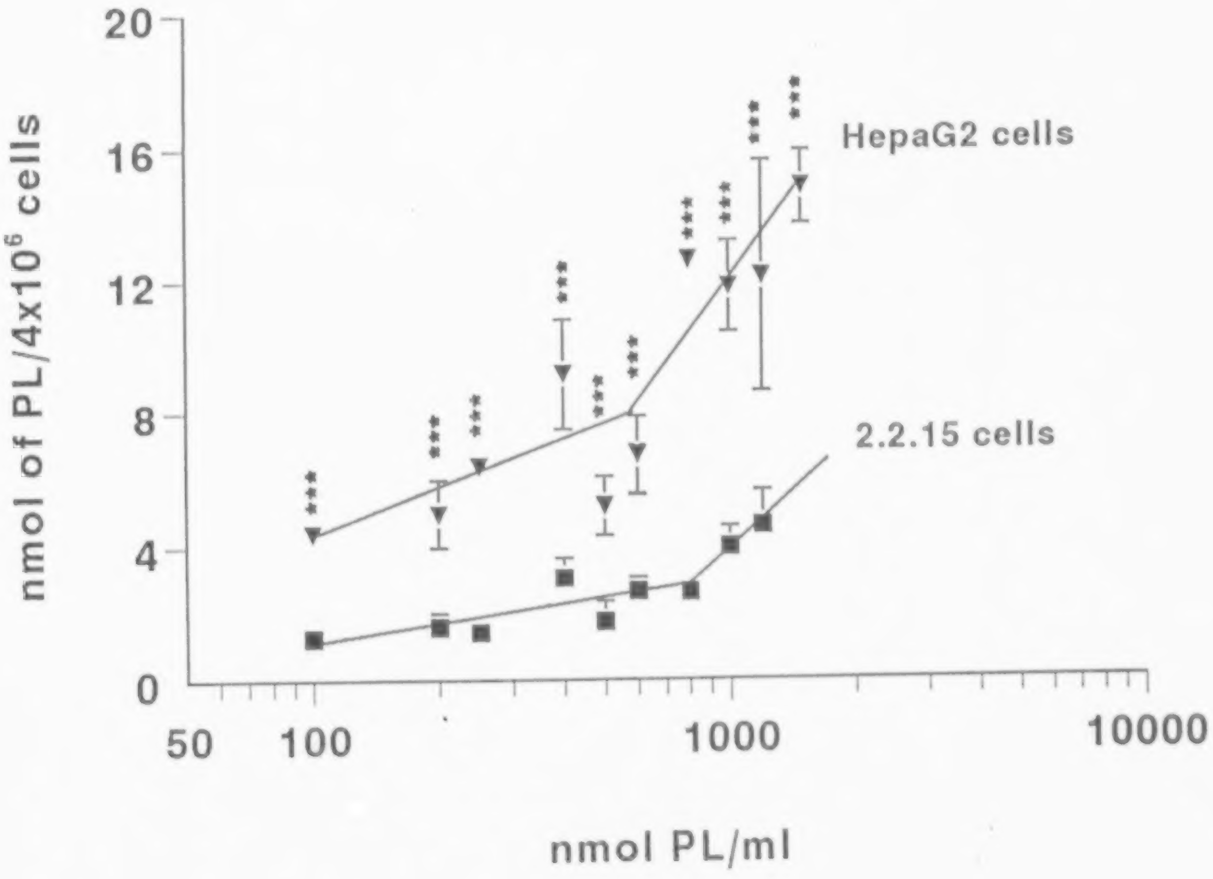
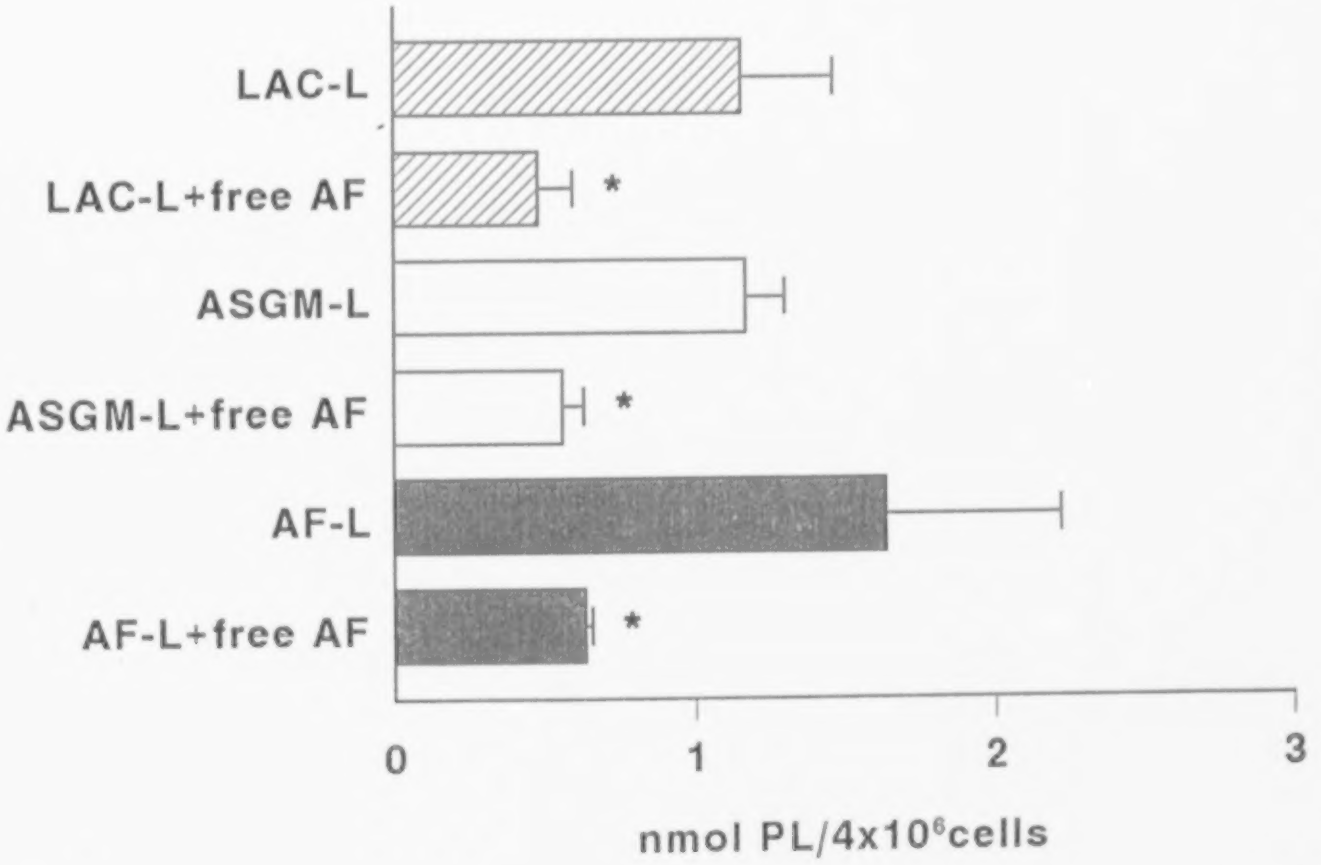


Figure 8 Competition experiments: Blockade of galactose ligand-liposome uptake by prior incubation of free asialofetuin with 2.2.15 cells.

Free asialofetuin solution (50 nmol/ml PBS, pH 7.4) was incubated with 2.2.15 cells prior to the treatment with liposome suspensions. After incubation with free asialofetuin at 37°C for 15 min., cells were treated with liposomes at 37°C for one more hour. Cells were washed, trypsinized and harvested as described in Materials and Methods. The data were calculated from the amount of ^{3}H-CHE-lipid label associating with 2.2.15 cells. Concentration of liposomes was 500 nmol phospholipid (PL)/ml. Data were from one experiment ($n=3$). Detail compositions of various liposomes are displayed in Table 1. Lactosylceramide- (LAC-) liposomes, striped bar; Asialo-GM$_1$- (ASGM$_1$-) liposomes, empty bar; Asialofetuin- (AF-) liposomes, shaded bar. Significant level (determined by t-test) indicates the difference between the uptake of each liposome type with and without prior incubation with free asialofetuin. ✱ p value ≤ 0.05.

Competitive Inhibition of Liposomal Uptake by Free Asialofetuin



galactose receptor ligands, was also observed by the HepG2 cell line with AF- > ASGM$_1$- ≥ LAC- > plain liposomes.

All three types of ligand-liposomes were much more easily taken up by HepG2 cells than by 2.2.15 cells, indicating that either the ß-galactose receptors are less exposed on the surface of 2.2.15 cells or the density of receptors on 2.2.15 cells is much lower than on the HepG2 cells.

It is also important to point out that the ligand-liposomes appear to be taken up by the cells in a biphasic manner (Fig. 2, Fig. 4), i.e., a critical concentration of phospholipid may be required to result in significantly increased ligand-liposomal uptake by 2.2.15 or HepG2 cells. While the phospholipid concentration was low, ligand-liposomes resulted only in a slightly increased uptake over plain liposomes, however, even this difference was statistically significant in the case of AF-liposomes or ASGM$_1$-liposomes. In comparison, after the phospholipid concentration reached a critical point, an increase in uptake was observed. This bi-phasic uptake of ligand-liposomes will be further discussed below.

(5) Liposomes Containing mAb against HBsAg

Unlike the above results seen for AF-liposomes at low phospholipid concentrations (5 or 10 nmol PL/ml), the uptake of mAb-liposomes by 2.2.15 cells showed no difference from that of plain liposomes (Fig. 9). At higher phospholipid concentrations (≥ 500 nmol PL/ml), the mAb-liposomes were taken up by 2.2.15 cells to a slightly higher degree than plain liposomes (Fig. 10, Table 5). This was statistically significant.

To investigate the specific uptake of mAb-liposomes, free mAb and sheep IgG, a non-recognized antibody, were used to compete with mAb-liposomes. The results of competition experiments with excess free mAb clearly showed that prior incubation with free mAb but not sheep IgG, blocked the uptake of mAb-liposomes by 2.2.15 cells (Fig. 11). These results indicated that the uptake of mAb-liposomes by 2.2.15 cells was via a specific receptor.

The uptake of mAb-liposomes by 2.2.15 cells was also compared with liposomes bearing different ß-galactose receptor ligands. The uptake of mAb-

Figure 9 Comparison of uptake, in 2.2.15 cells, of mAb-liposomes (HSPC:CH:MPB-PE, 2:1:0.02 molar ratio) and ligand-free liposomes (HSPC:CH, 2:1 molar ratio) at low phospholipid concentrations.

Liposomes were conjugated to mAb against HBsAg as described in Materials and Methods. Open bar indicates 5 nmol PL/ml; shaded bar indicates 10 nmol PL/ml. Cells were incubated with liposomes at 37°C for one hour. PL, phospholipid; Plain-L, plain liposomes; mAb-L, liposomes conjugated to mAb-against-HBsAg.

Uptake of mAb-Liposomes by 2.2.15 Cells
Compared with Plain Liposomes

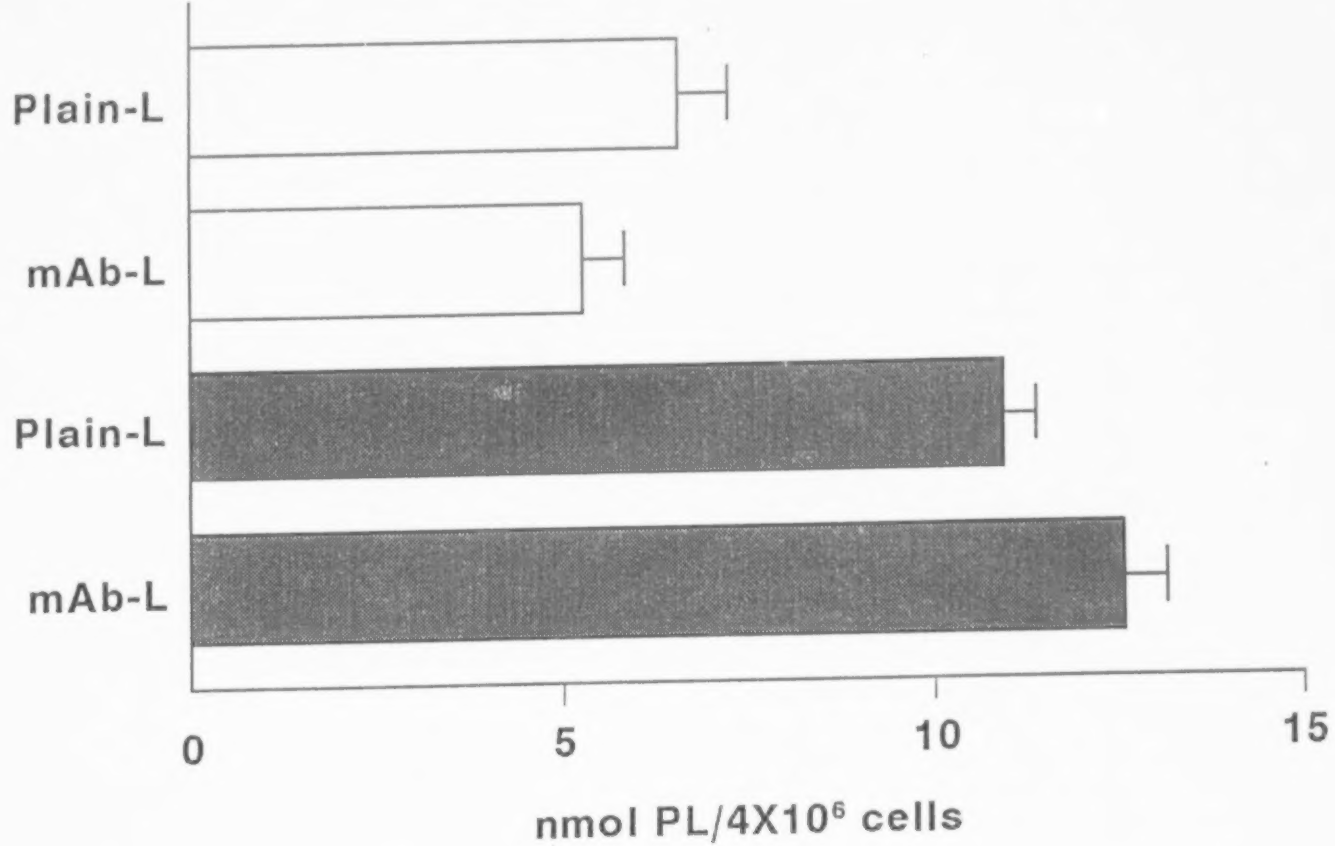


Figure 10 Comparison of uptake, in 2.2.15 cells, of mAb-liposomes (HSPC:CH:MPB-PE, 2:1:0.02 molar ratio) with ligand-free liposomes (HSPC:CH, 2:1 molar ratio).

Cells were incubated with increasing concentrations of liposome suspensions at 37°C for one hour. Trypsinization procedure was as described in Materials and Methods. Data were from three separate experiments ($n=9$). Plain liposomes (●); mAb-liposomes, liposomes conjugated to mAb-against-HBsAg (◆). Significant level (determined by t-test) indicates the difference between the uptake of plain and mAb-liposomes. ** p value≤ 0.01.

Uptake of mAb-liposomes
by 2.2.15 Cells

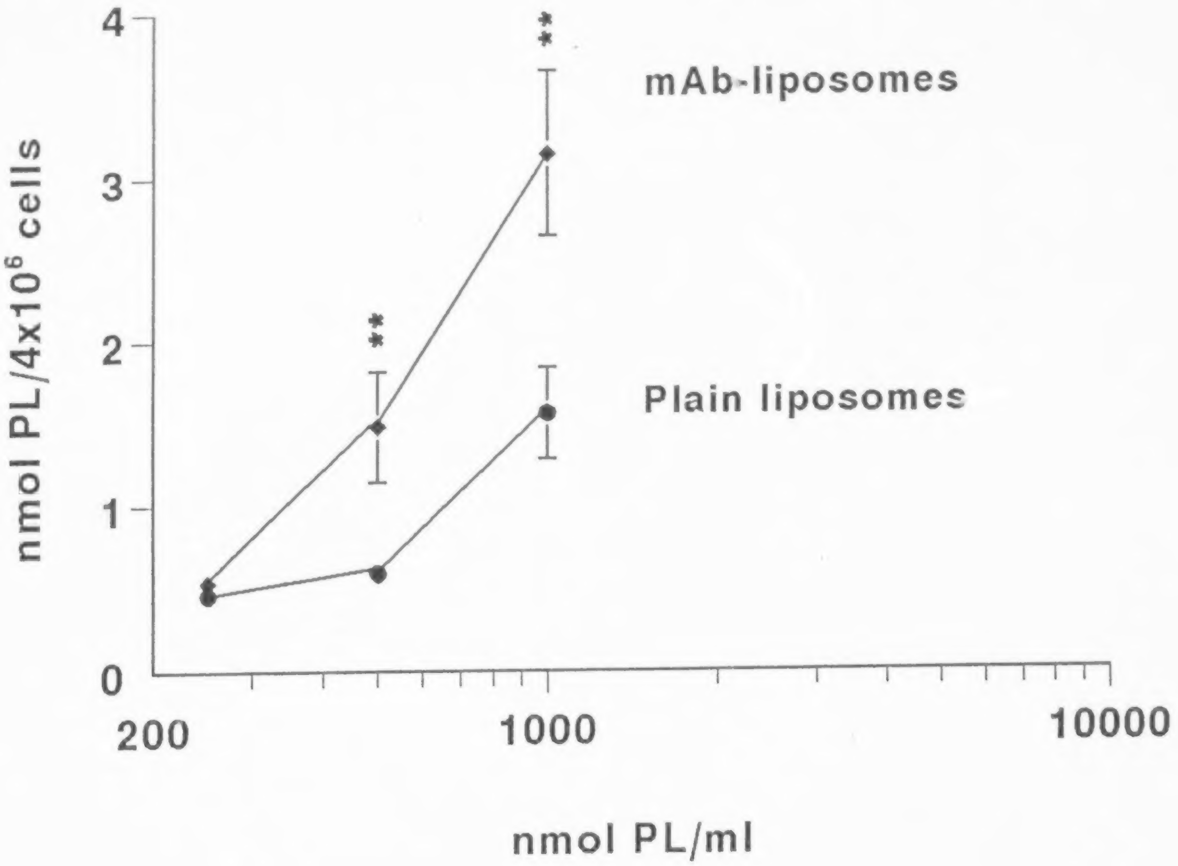
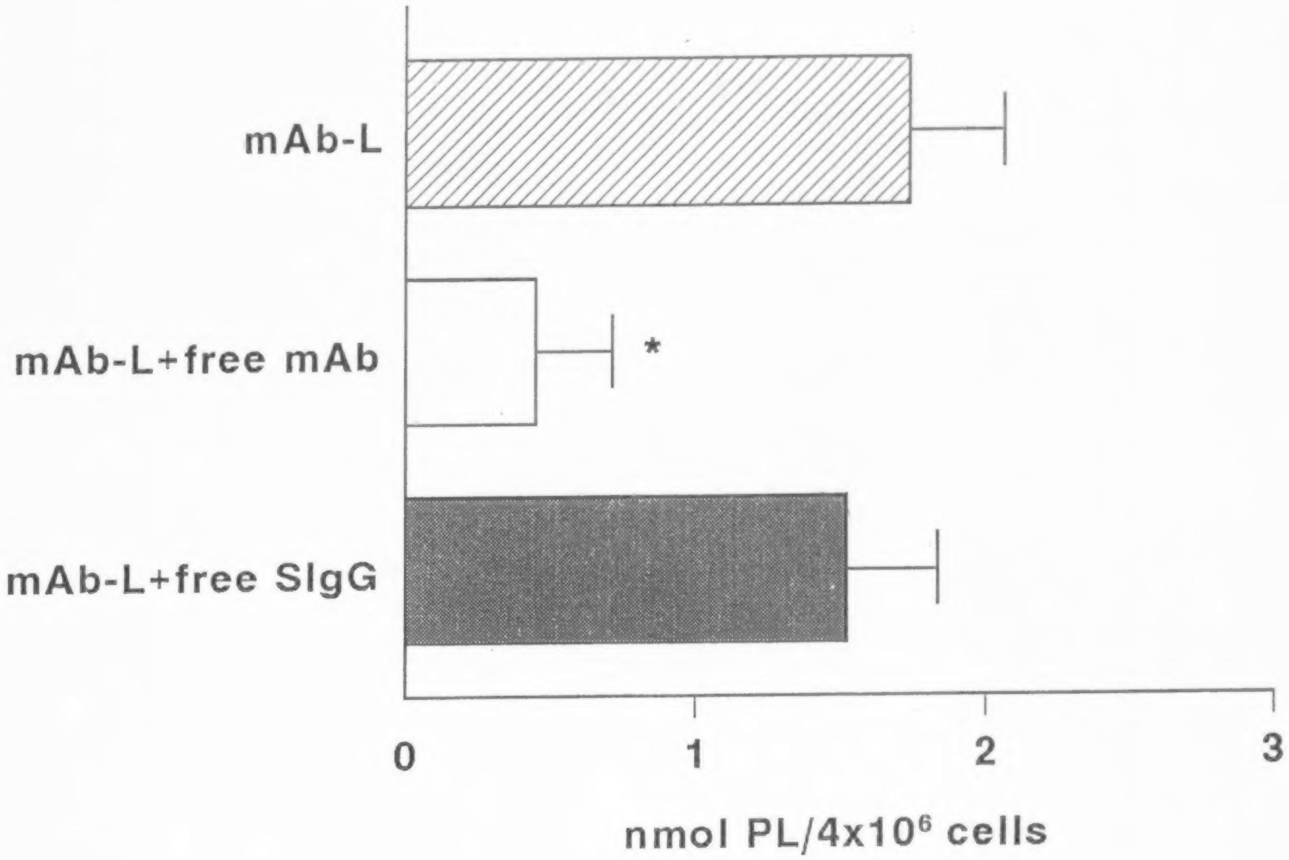


Figure 11 Competition experiments: Blockade of uptake of mAb-liposomes (HSPC:CH:MPB-PE, 2:1:0.02 molar ratio) by prior incubation of free mAb or free sheep-IgG (0.6 mg/ml PBS, pH 7.4) with 2.2.15 cells .

2.2.15 cells were first incubated with free mAb or free sheep IgG solution. After incubation at 37°C for 15 min., cells were further incubated with mAb-liposomes for one more hour. Trypsinization procedure was as described in Materials and Methods. Data were obtained from one experiment (n = 3). mAb, mAb-against-HBsAg; mAb-L, liposomes conjugated to liposomes. Striped bar, uptake of mAb-liposomes; empty bar, uptake of mAb-liposomes with prior incubation with free mAb; shaded bar, uptake of mAb-liposomes with prior incubation with free sheep IgG (SIgG). Significant level (determined by t-test) indicates the difference between the uptake of mAb-liposomes with and without prior incubation of free mAb. ✱ p value ≤ 0.05.

Competitive Inhibition of mAb-liposome
Uptake by Free mAb or Free SIgG



liposomes is intermediate between that of AF-liposomes or plain liposomes and was in the same range as LAC-liposomes and ASGM$_1$-liposomes (Table 5). The increased uptake of mAb-liposomes, LAC-liposomes or ASGM$_1$-liposomes at 500 or 1000 nmol PL/ml by 2.2.15 cell are statistically significant (t-test, p value$\leq$0.01), as compare to plain liposomes. In the HepG2 cell line, the uptakes of mAb-liposomes at high PL concentrations (1.0 ± 0.2 and 2.1 ± 0.7 nmol PL/4x10^6 cells, at 500 and 1000 nmol PL/ml, respectively) were indistinguishable from those of plain liposomes (1.0 ± 0.1 and 1.4 ± 0.2 nmol PL/4x10^6 cells, at 500 and 1000 nmol PL/ml, respectively) (Table 5).

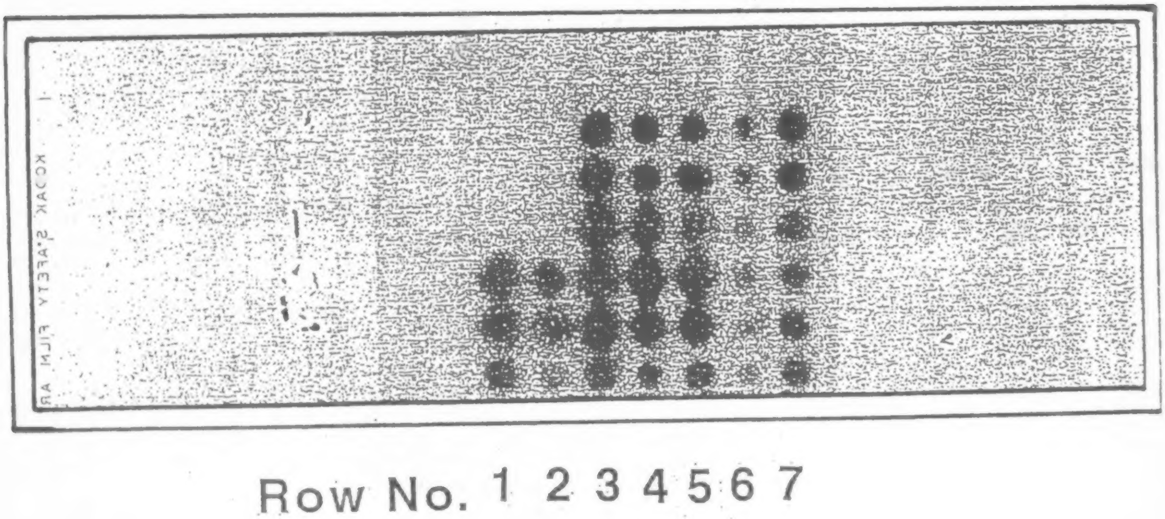
(C) *Inhibition of HBV Replication by Liposomal ddGTP*

The inhibition of HBV replication was evaluated by measuring the decrease in levels of HBV-DNA. Figure 12 shows the result of a typical dot blotting experiment after Nick translation. The intensity of each spot on the X-ray film image reflects the concentration of HBV-DNA, i.e. the darker the image, the higher the HBV-DNA concentration and lower the inhibition of HBV-DNA replication. It seems that AF-liposomes containing ddGTP (row 5) were not effective in inhibiting HBV-DNA replication in 2.2.15 cells. A similar result was seen in the case of plain liposomes with entrapped ddGTP (row 4). Treating transfected cells with free ddGTP in PBS (row 3) also did not inhibit the replication of DNA.

Empty liposomes (both plain and AF-liposomes; row 1 and 2, respectively) did not inhibit HBV-DNA replication, suggesting that the liposomes themselves are unable either to inhibit viral DNA replication or kill transfected hepatoma cells, since the loss of 2.2.15 cells (decrease in numbers of host cells) also would result in decreased amounts of HBV-DNA. According to the results obtained, ddGTP-containing liposomes can neither cure nor kill 2.2.15 cells.

Figure 12 Inhibition of HBV replication in 2.2.15 cells by liposomal ddGTP or free ddGTP.

2.2.15 cells were treated with empty plain liposomes (1000 nmol PL/ml) (row 1), empty AF-liposomes (no drug) (1000 nmol PL/ml) (row 2), free ddGTP solution (250 μg/ml PBS, pH 7.4) (row 3), plain liposomes loaded with ddGTP (1000 nmol PL/ml) (row 4) and AF-liposomes loaded with ddGTP (1000 nmol PL/ml) (row 5) by a fourteen-day protocol as described in Materials and Methods. After fourteen days, cells were digested and cellular DNA with incorporated HBV DNA was extracted. By using nick translation and dot blotting techniques, the HBV DNA segments incorporated into the cellular DNA of 2.2.15 cells was labelled with ^{32}P. Thus, the existence of HBV DNA was converted into the presence of ^{32}P radioactivity. The blotted samples were imaged as described in Materials and Methods. The intensity of each sample image is proportional to the amount of ^{32}P which is further related to the amount of HBV DNA remaining in 2.2.15 cells after the fourteen-day treatment. Row 6 is a negative control (HepG2 cells) and row 7 is a positive control (untreated 2.2.15 cells). Each row consists of identical, replicate samples (n = 3 or 6).


KODAK SAFETY FILM AR
Row No. 1 2 3 4 5 6 7

(D) *Internalization of Liposomes by HBV-transfected Hepatoma Cells*

Internalization of liposomes was investigated by using the HPTS method described by Daleke et al. (1990). HPTS is a pH-sensitive fluorescent material and is not permeable through liposomes after encapsulation. After excitation, HPTS shows two major emission peaks (at wavelength of 403 nm and 450 nm) which are pH-dependent. It was found that the emission fluorescence of HPTS decreased at 403 nm and increased at 450 nm with an increase in the pH of its environment (Daleke et al.,1990). By contrast, with a decrease in pH, the emission at 403 nm increases and that at 450 nm decreases (when the pH value is lower than 5, the emission peak at 450 nm will totally vanish). The emission at 413 nm is pH independent, i.e., changes in environmental pH do not change the fluorescent intensity emitted at 413 nm. The emission intensity at 413 nm thus provides a direct measure of the HPTS concentration and increases with the increasing HPTS concentration, when other experimental conditions are identical. This pH-independent emission at 413 nm can also be used to normalize measurements for changes in HPTS content. It has been determined by Daleke and coworkers that the ratio of HPTS emission intensity at 403 nm to 413 nm (403 nm/413 nm) is a constant with a value of approximate 2 when the environmental pH is lower than 6. The value of the fluorescence ratio at 403 nm to 413 nm decreases steadily as the environmental pH increases, reaching a value of approximately 0.8 at pH 9.

The relationship between the ratio of HPTS emission at 450 nm to that at 413 nm (450 nm/413 nm) also varies with environmental pH. When the environmental pH is lower than 5, the value of 450 nm/413 nm is zero (no emission peak at 450 nm when pH is lower than 5), and this ratio steadily increases with increasing pH, reaching a value of approximately 3 at pH 8.0 (Daleke et al., 1990) (see Table 6). Accordingly, one can record the emission profile of HPTS and calculate the 403 nm/413 nm and 450 nm/413 nm values which can be used to estimate the pH value of environment to which HPTS is exposed.

In preliminary experiments, 1 μM HPTS in PBS was adjusted to various pH.

Table 6 Relationship between fluorescent emission intensity of the pH-sensitive probe, HPTS, and changes of environmental pH.

(Summary of the study by Daleke et al., 1990)

Environmental pH	**Ratio of emission intensity**	
pH value	403 nm/413 nm	450 nm/413 nm
below 5	2	0
5 → 6	2	0 → 0.2
6 → 7	2 → 1.5	0.2 → 1.5
7 → 8	1.5 → 1	1.5 → 3
8 → 9	1 → 0.8	3 → 4

Figure 13 Emission profiles of HPTS (1 μM in PBS) at pH 5, 6 and 7.

1 μM HPTS in PBS was adjusted to various pH values (pH 5,6 and 7) and slit width for excitation and emission were set as 1.8 and 4.5 nm, respectively. Fluorescent intensity is expressed as peak height (in centimeter, CM). Experiments were performed as described in Materials and Methods. **A**. pH 5; **B**. pH 6; **C**. pH 7.

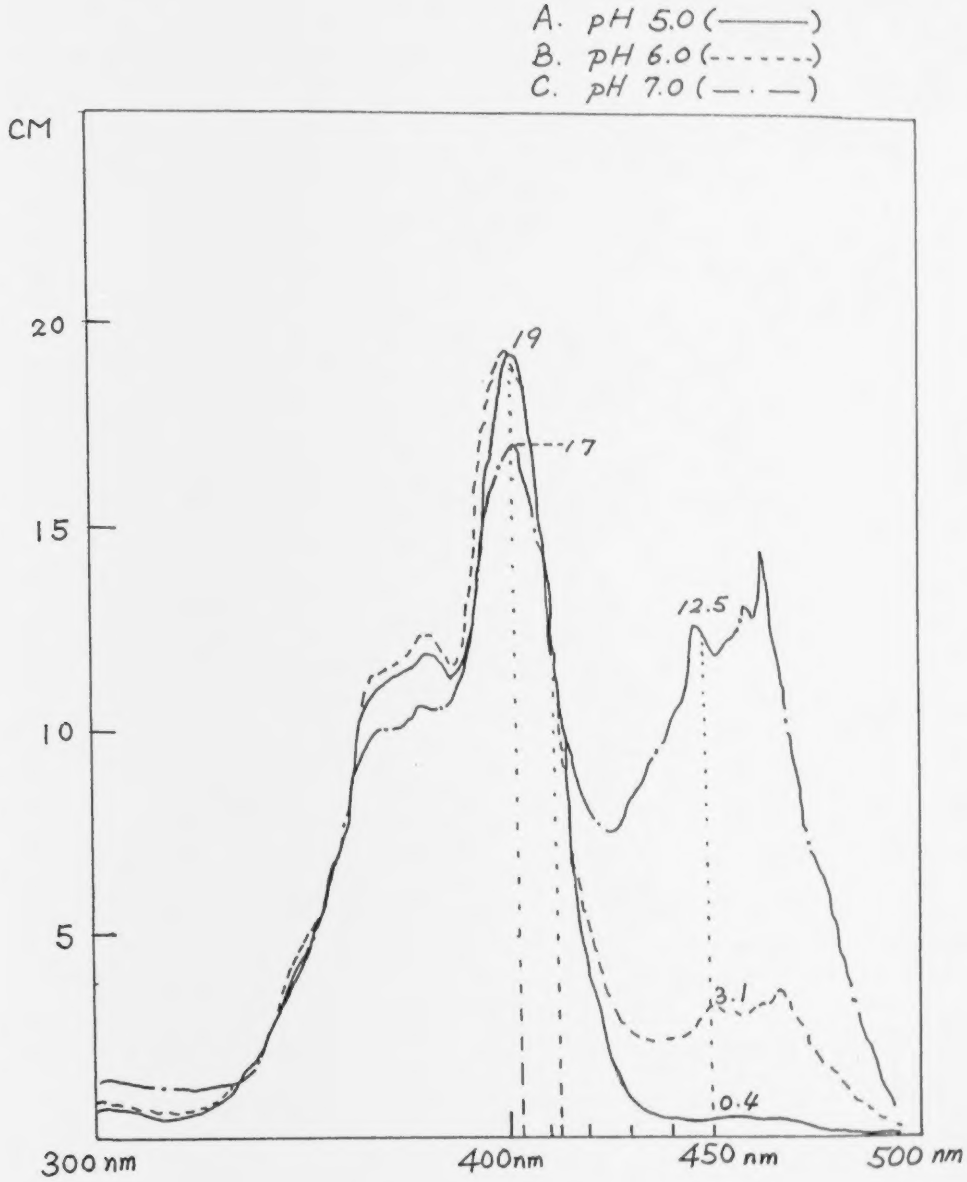

A. pH 5.0 (———)
B. pH 6.0 (- - - - - - -)
C. pH 7.0 (— · —)
CM
20
15
10
5
19
17
12.5
3.1
0.4
300 nm
400nm
450 nm
500 nm

Figure 13 A-C show the profiles of fluorescent emission at pH 5, 6 and 7, respectively for 1 μM HPTS in PBS. Slit widths for excitation and emission were set as 1.8 and 4.5 nm, respectively. Fluorescent intensity is expressed as peak height in centimeter (CM). It was found that these profiles matched the patterns described by Daleke and coworkers (1990). The peak heights at 413 nm of pH 5, 6 and 7 were 12.1, 12.1, 12.2 cm, respectively. This supports previous data values for pH 5, 6 and 7, demonstrating that the emission of HPTS at 413 nm is pH-independent. Ratio of 403 nm/413 nm and 450 nm/413 nm are calculated. The values for the fluorescent ratio 403 nm/413 nm for pH 5, 6 and 7 are 1.6, 1.6 and 1.4, respectively. The values for the ratio of 450 nm/413 nm for pH 5, 6 and 7 are 0.03, 0.26 and 1.02, respectively. These numbers are consistent with those reported by Daleke et al. (1990).

Internalization experiments were performed and the scanned emission profiles (from 300 to 500 nm) of HPTS, encapsulated in AF-liposomes or plain liposomes, were obtained. These profiles are shown in Figure 14. The pH-dependent intensity of emission (at 403 nm or 450 nm) and the pH-independent emission (at 413 nm) was measured. The 403 nm/413 nm and 450 nm/413 nm values were calculated (Table 7).

After a twenty-four-hour incubation in the presence of 5% CO_2 at 37°C, the 403 nm/413 nm and 450 nm/413 nm values of AF-liposomal HPTS taken up by 2.2.15 cells were 1.4 and 0.95 respectively. As can be seen from Table 6, these two figures indicate that the pH value is between 6 and 7 and very close to 7. A reasonable estimation was made as 6.9 (Table 7). For HPTS in plain liposomes, incubated with 2.2.15 cells at 37°C for 24 hours, the 403 nm/413 nm and 450 nm/413 nm values are 1.6 and 0.53, respectively, indicating that the environmental pH is between 6 and 6.5 (Table 7). After incubation for two hours, the two ratios of AF-liposomal HPTS were 2 and 0.03 for 403 nm/413 nm and 450 nm/413 nm respectively. These indicate that the environmental pH is between 5 and 6 and closer to 6. It is estimated that the environmental pH in this case is between 5.5 and 6 (Table 7).

Figure 14 Internalization of liposomes containing HPTS by 2.2.15 cells.

AF-liposomes (or those lacking AF) containing HPTS were incubated with 2.2.15 cells at 37°C for two or twenty-four hours. After the incubation, treated cells were washed, trypsinized and resuspended at a concentration of 2×10^5 cell/ml PBS. The pH-dependent property of HPTS was used to monitor the incorporation of fluorescent liposomes into cell compartments with different pHs. The emission profile of HPTS loaded into AF-liposomes incubated at 37°C for two hours (AF/2hr) is indicated as a continuous line (——), and that for 24 hours (AF/24hr) as a dotted line (-----). The emission profile of HTPS loaded into plain liposomes, incubated at 37°C for twenty-four hours (PL/24hr), is indicated as a dot-interrupted line (——•——). Fluorescent intensity was expressed as peak height (in centimeter, CM). Ratio of 403 nm/ 413 nm and 450 nm/ 413 nm were calculated in every case. These ratio values were further converted into pH values as described by Daleke et al. (1990). AF, asialofetuin.

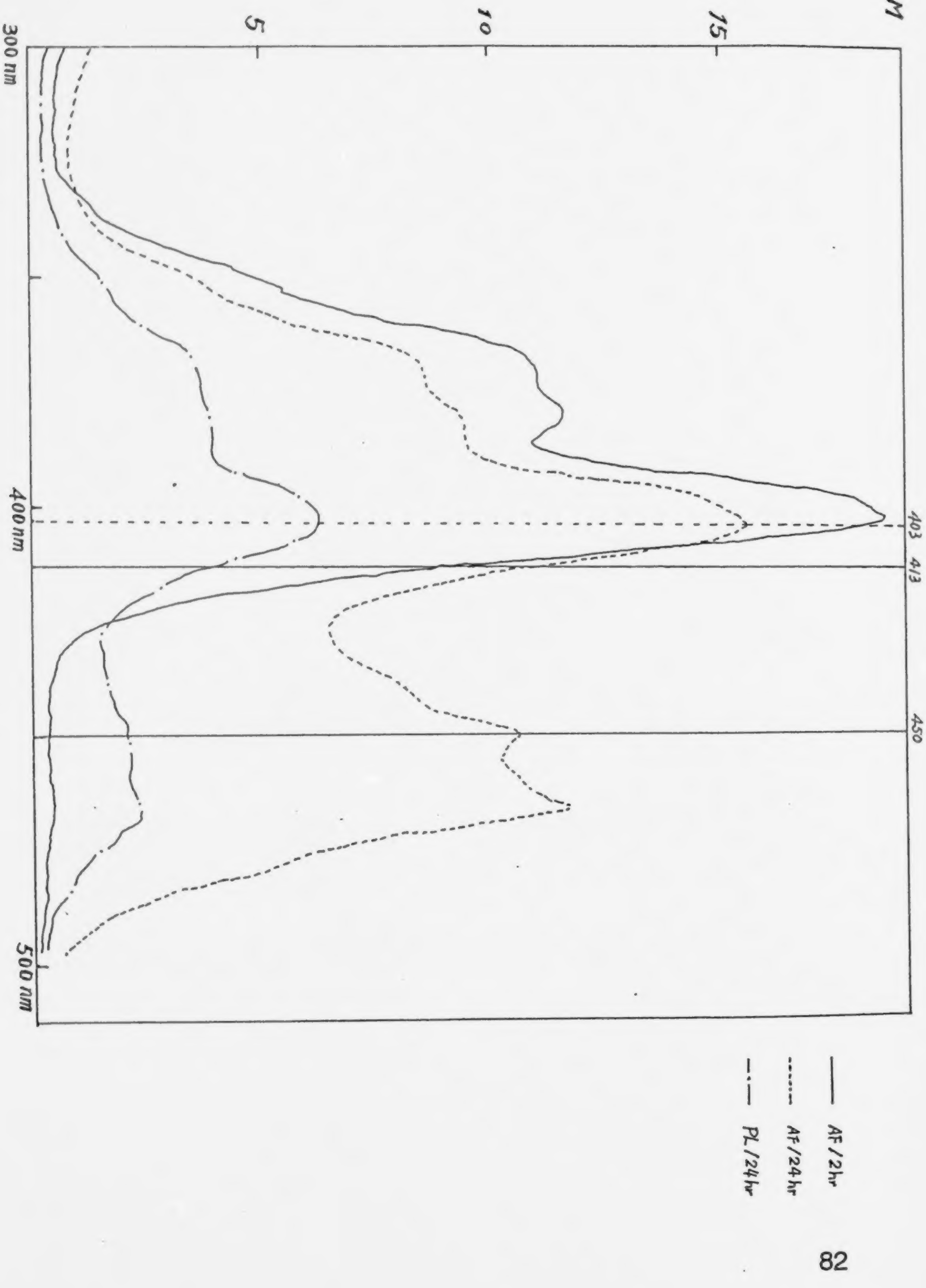

CM
15
10
5
300 nm
400 nm
500 nm
403
413
450
AF / 2hr
AF / 24hr
PL / 24hr

Table 7 Internalization of AF-liposomes containing HPTS by 2.2.15 cells.

The internalization of liposomes was monitored by encapsulation of HPTS into AF-liposomes [AF-HSPC:CH (2:1)] or plain liposomes [HSPC:CH (2:1)] to detect the pH of the environment to which AF- or plain liposomes were exposed. The intensity of fluorescent emission was measured as peak height in centimetres (CM) which was further used to calculate the 403 nm/413 nm and 450 nm/413 nm values. These two parameters were converted into pH values according to Table 6 suggested by Daleke et al. (1990).

	Ratio of fluorescent intensity on different emission wavelength		**pH independent emission**	**Estimated pH**
	403 nm/413 nm	450 nm/413 nm	413 nm (CM)	pH
AF-HSPC:CH (2:1) after incubation at 37^{0}C for 24 hours	1.4	0.95	11	6.9
HSPC:CH (2:1) after incubation at 37^{0}C for 24 hours	1.6	0.53	3.8	6.0 - 6.5
AF-HSPC:CH (2:1) after incubation at 37^{0}C for 2 hours	2.0	0.03	8.8	5.5 - 6.0
	$413_{AF24}/413_{PL24} = 2.89$			

These decreases in pH (from the intra-liposomal pH of 7.4 to environmental pH 5.5, 6 or 6.9 detected after incubations under different conditions) indicate the internalization of AF- and plain liposomes by 2.2.15 cells because the extracellular pH is 7.4 and intracellular pH is lower than 7. The ratio of pH-independent emission of AF-liposomes to that of plain liposomes ($413_{AF24}/413_{PL24}$) is 2.89 (Table 7). Since the cell numbers sampled (2×10^5 2.2.15 cells per ml PBS), slit width of emission and excitation are identical for each sample, ratio of fluorescence intensities emitted at 413 nm can be used to provide an estimate of the amount of liposomes associating with cells. Thus the value of $413_{AF24}/413_{PL24}$ of 2.89 reflects the difference in uptake and internalization between AF-liposomes and plain liposomes. Higher $413_{AF24}/413_{PL24}$ values indicate higher uptake and internalization of AF-liposomes than plain liposomes. Thus a value of $413_{AF24}/413_{PL24}$ of 2.89 suggests that more AF-liposomes were associating with 2.2.15 cells than were plain liposomes after incubation at 37°C for 24 hours, providing an independent confirmation of the uptake data for AF-liposomes obtained with radiolabelled liposomes.

After incubation for 2 hours, the environmental pH of AF-liposomes is estimated to be 5.5-6, indicating that AF-liposomes are likely located in endosomes and/or lysosomes well known to have low pH values (Barrett et al., 1983). After incubation for 24 hours, the pH of AF-liposomal HPTS taken up by 2.2.15 cells increases to 6.9. This indicates that AF-liposomal HPTS has been transported into a cytosolic apparatus with a higher pH environment (possibly Golgi apparatus), rather than remaining in lysosomes or endosomes with a lower pH value of 5.5-6. In comparison, after twenty-four-hour incubation, the plain liposomal HPTS most likely remains in lysosomes or endosomes. These results suggest some possible differences in the metabolic fate and intra-cellular transportation pathways between AF-liposomes and plain liposomes which will be discussed later.

(E) *In Vivo* *Study of Various Liposomes (with or without PEG) and Their Intrahepatic Distribution*

Table 8A shows the average diameter of each type of liposomes used in the *in* *vivo* studies. The conjugation ratio of asialofetuin on liposomes (with or without PEG) is shown in Table 8B. The initial diameter was controlled to ≤ 85 nm in every case. After conjugation, the average diameter of AF-liposomes was approximately 15 nm larger than the initial diameter. This result was comparable with that of *in vitro* experiments (Table 2). In comparison, the average diameter of AF-PEG-liposomes was approximately 5 nm larger than the diameter before protein conjugation (Table 8A), indicating that the total length of the asialofetuin-biotin-avidin complex was either only slightly longer than, or almost the same as, that of a PEG-chain.

The clearance from blood, and half-life in the circulatory system, of various liposomes are shown in Fig. 15 A,B,C, and D. The incorporation of PEG contributed to slightly longer liposomal lifespan in circulation ($T_{1/2}$ of PEG-liposomes > AF-PEG-liposomes > Plain liposomes ≥ AF-liposomes; Table 9) and slightly decreased liver uptake. It was found that, while the liver uptake decreased by 6%, on average, the presence of PEG did not alter the spleen uptake significantly (Table 10). These results indicated that the addition of PEG to liposomes (with or without asialofetuin) contributed to a slightly longer half-life in the circulatory system ($T_{1/2}$ = 22-26 hours; Table 9), as compared to liposomes lacking PEG ($T_{1/2}$ = 18-19 hours; Table 9). The half-lives of AF-liposomes and plain liposomes were similar at 18.2 hours and 18.6 hours, respectively. Additionally, the half-life of AF-PEG-liposomes was found to be 22 hours, slightly shorter than that of PEG-liposomes (25.6 hours). These results indicated that conjugation of asialofetuin to the liposomal surfaces (with or without PEG) only slightly shortened their lifespan in the circulatory system (Table 9), as compared to ligand-free PEG-liposomes or ligand-free liposomes.

As shown in Fig. 16A and Table 11, the uptake of HSPC:CH liposomes after conjugating to asialofetuin (AF-HSPC:CH), was shifted by about 30% from Kupffer cells to parenchymal cells, suggesting that conjugation of asialofetuin on HSPC:CH liposomes contributed to a higher uptake by parenchymal cells than Kupffer cells. Incorporation of PEG into HSPC:CH liposomes resulted in a 14% shift from Kupffer cells to parenchymal cells, compared with liposomes lacking PEG (Fig. 16B, Table 11).

Table 8A Average diameter of various liposomes used in *in vivo* studies.

Liposome diameters (mean ± S.D., n=3) were determined by measuring their dynamic light scattering as described in Materials and Methods.

Composition	Average diameter	Initial diameter
HSPC:CH (2:1)	77.4 ± 2.7 nm (n=3)	ND
PEG-HSPC:CH (2:1)	83.6, 84 nm	ND
AF-HSPC:CH (2:1)	91, 94 nm	74.9,76 nm
AF-PEG-HSPC:CH (2:1)	84, 85 nm	80, 80 nm

ND: not determined

Table 8B Conjugation ratio of asialofetuin to the HSPC:CH and PEG-HSPC:CH liposomes used in *in vivo* studies.

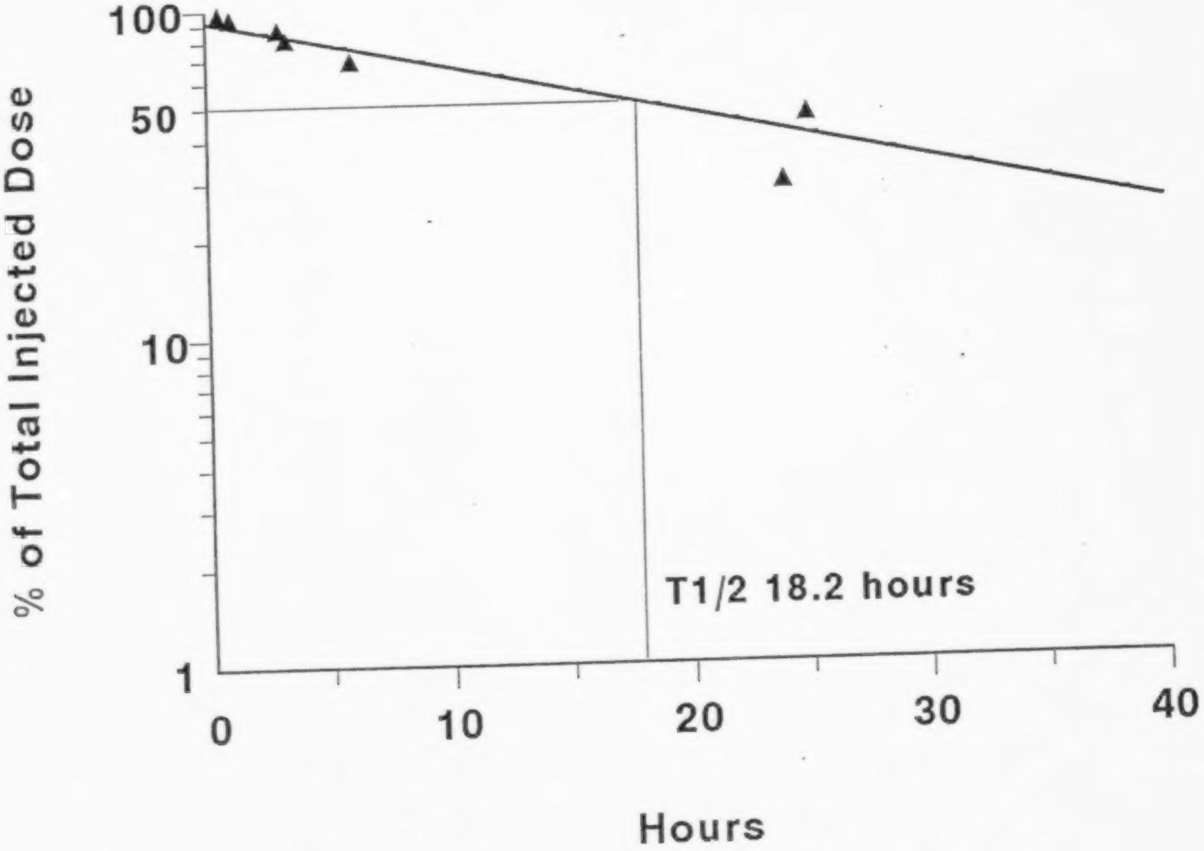
The conjugation ratio of asialofetuin (AF) to phospholipid was determined by using modified Lowry assay to measure the appearance of AF on the liposomal surfaces as described in Materials and Methods.

Composition	μmol AF/μmol PL
AF-HSPC:CH (2:1)	2.28×10^{-4}
AF-PEG-HSPC:CH (2:1)	4.56×10^{-4}

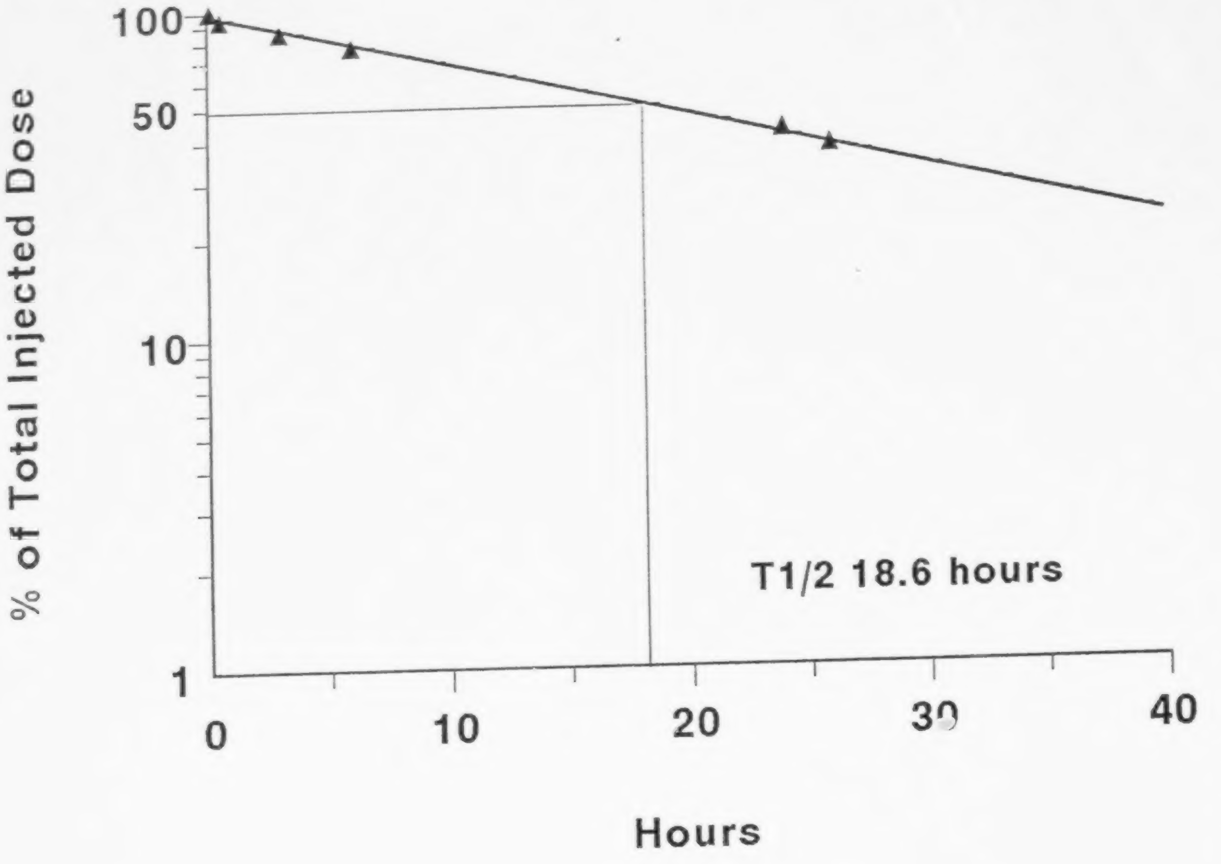
Figure 15 Rate of blood clearance of various liposomes in rats.

Four different compositions of liposomes labelled with ^{3}H-COE-lipid, were injected into male Wag/Rij rats by the intravenous route (penis vein). Rats received 3 μmol of liposomal phospholipid in less than 1 ml of HEPES buffered saline (pH 7.4). Blood samples were taken from the tail vein at different time points after injection. By comparing the radioactivity remaining in the circulation, the percentages of various liposomes remaining in blood at different time points (▲) were determined. Half-life of each liposome type was calculated from the clearance rate. **A**. AF-HSPC:CH (2:1); **B**. HSPC:CH (2:1); **C**. AF-PEG-HSPC:CH (2:1); **D**. PEG-HSPC:CH (2:1).

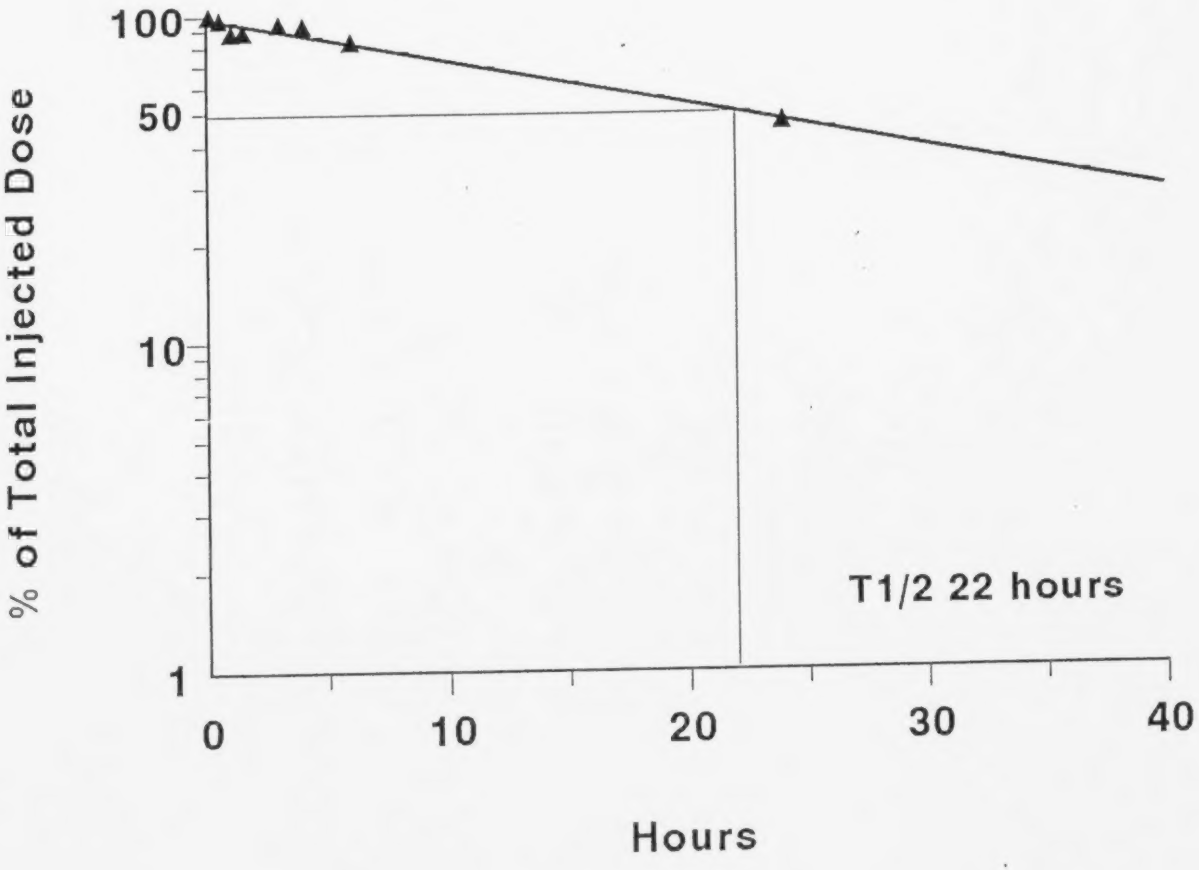
A. Clearance of AF-HSPC:CH (2:1) Liposomes from Blood in Rats



B. Clearance of HSPC:CH (2:1) liposomes from Blood in Rats



C. Clearance of AF-PEG-HSPC:CH (2:1) Liposomes from Blood in Rats



D. Clearance of PEG-HSPC:CH (2:1) Liposomes from Blood in Rats

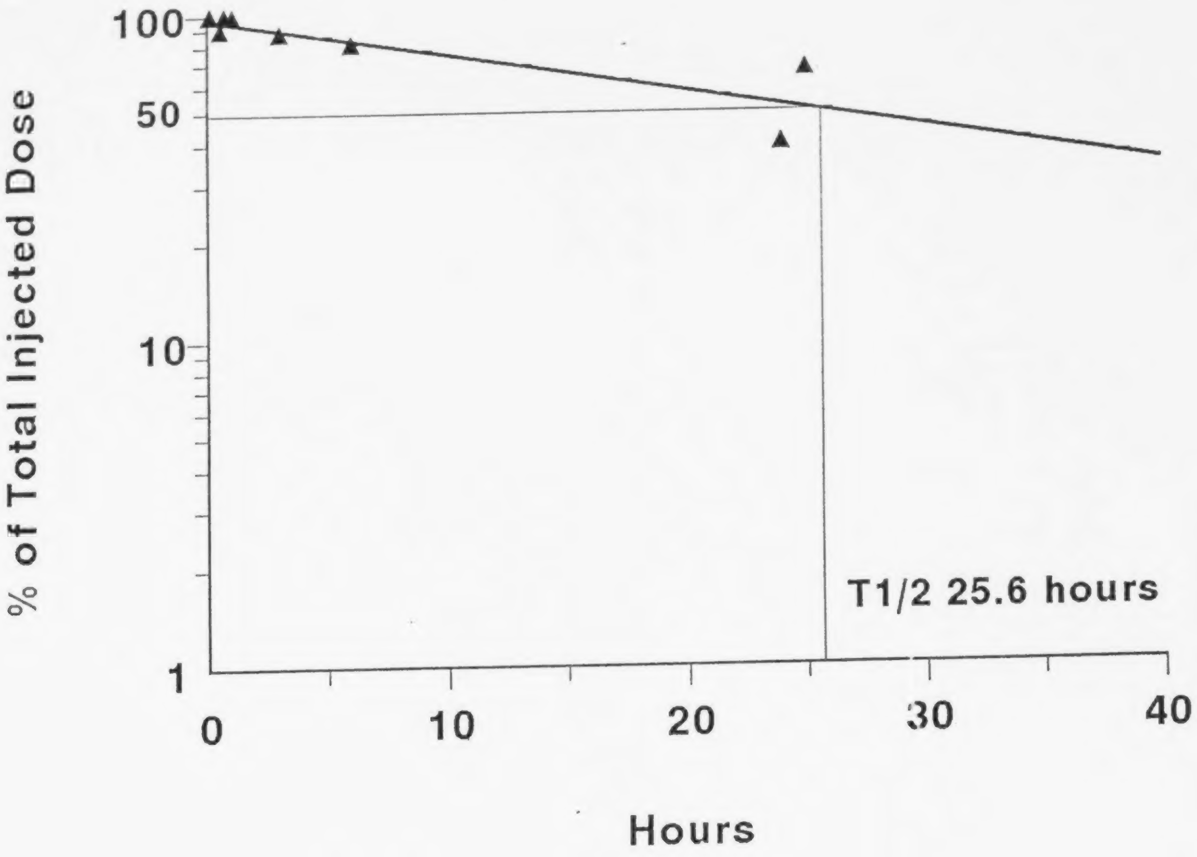


Table 9 Half-life of blood clearance of various liposomes in rats.

Four different compositions of liposomes labelled with ^{3}H-COE-lipid, were injected into male Wag/Rij rats by the intravenous route (penis vein). Rats received 3 μmol of liposomal phospholipid in less than 1 ml of HEPES buffered saline (pH 7.4). Blood samples were taken from the tail vein at different time points after the injection. By comparing the radioactivity remaining in the circulation, the percentages of various liposomes remaining in blood at different time points were determined. Half-life of each liposome type was calculated from the clearance rate.

Composition	Half-life of clearance (Hours)
AF-HSPC:CH (2:1)	18.2
HSPC:CH (2:1)	18.6
AF-PEG-HSPC:CH (2:1)	22
PEG-HSPC:CH (2:1)	25.6

Table 10 Liver and spleen uptake of various liposomes in rats.

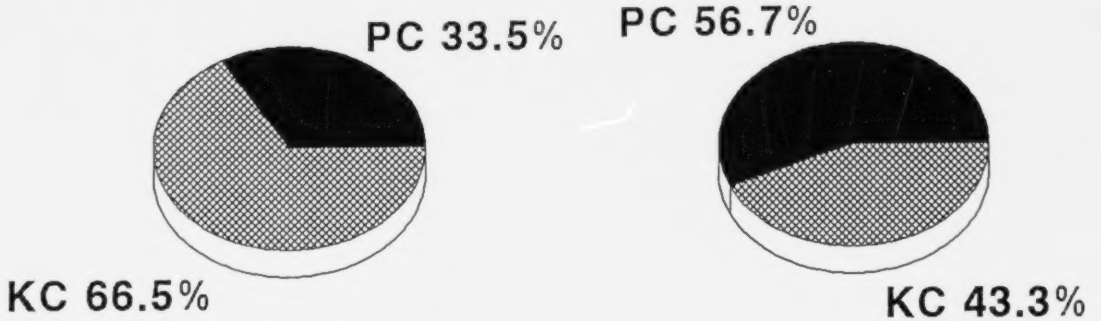
Four different compositions of liposomes labelled with ^{3}H-COE-lipid were injected into male Wag/Rij rats (n = 1-3), by the intravenous route (penis vein). Rats received 3 μmol of liposomal phospholipid in less than 1 ml of HEPES buffer saline (pH 7.4). At 24 hours after the injection, the animal was anaesthetized and sacrificed. Total liver or spleen uptake were determined by measurement of ^{3}H-COE-lipid label recovered in liver or spleen and expressed as the percentage to the total injected dose. Mean ± S.D. for HSPC:CH (n = 3). Recovery of injected radio-label from the circulatory system, liver, and spleen was found to be approximately 70% of the total injected dose in average, at 24-hour post-injection.

Composition	Hours after injection	liver (%)	spleen (%)
HSPC:CH (2:1)	24	17.6 ± 2.2	5.9 ± 1.0
PEG-HSPC:CH (2:1)	24	15.6, 8.3	5.8, 6.6
AF-PEG-HSPC:CH (2:1)	24	6.5, 16	3, 6.7
AF-HSPC:CH (2:1)	24	19.6	12.6

Figure 16 Intrahepatic distribution of various liposomes in rats.

Four different compositions of liposomes labelled with ^{3}H-COE-lipid were injected into male Wag/Rij rats, by the intravenous route (penis vein). Rats received 3 μmol of liposomal phospholipid in less than 1 ml of HEPES buffer saline (pH 7.4). At 24 hours after the injection, the animal was anaesthetized and sacrificed. Liver perfusion and isolation of parenchymal cells (PC) and Kupffer cells (KC) were performed as described in Materials and Methods. Liver distribution was expressed as the percentage of various liposomes recovered in a specific cell population (e.g. PC or KC) relative to the total liver uptake of that particular liposome type. **A**. HSPC:CH and AF-HSPC:CH liposomes; **B**. PEG-HSPC:CH and HSPC:CH liposomes; **C**. PEG-HSPC:CH and AF-PEG-HSPC:CH liposomes.

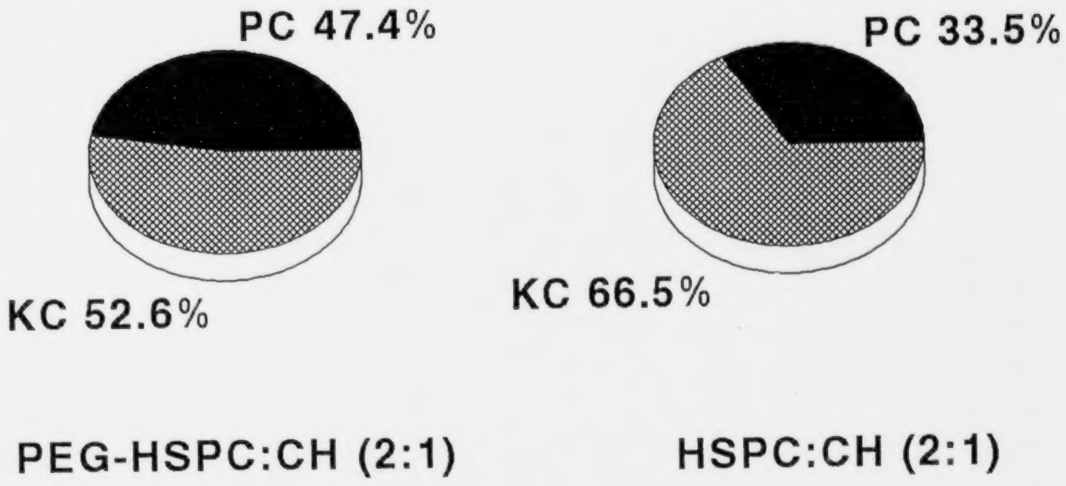
A. Intrahepatic Distribution of HSPC:CH (2:1) Liposomes



HSPC:CH (2:1)

AF-HSPC:CH (2:1)

B. Intrahepatic Distribution of Liposomes with or without PEG



C. Intrahepatic Distribution of
PEG-HSPC:CH (2:1) Liposomes

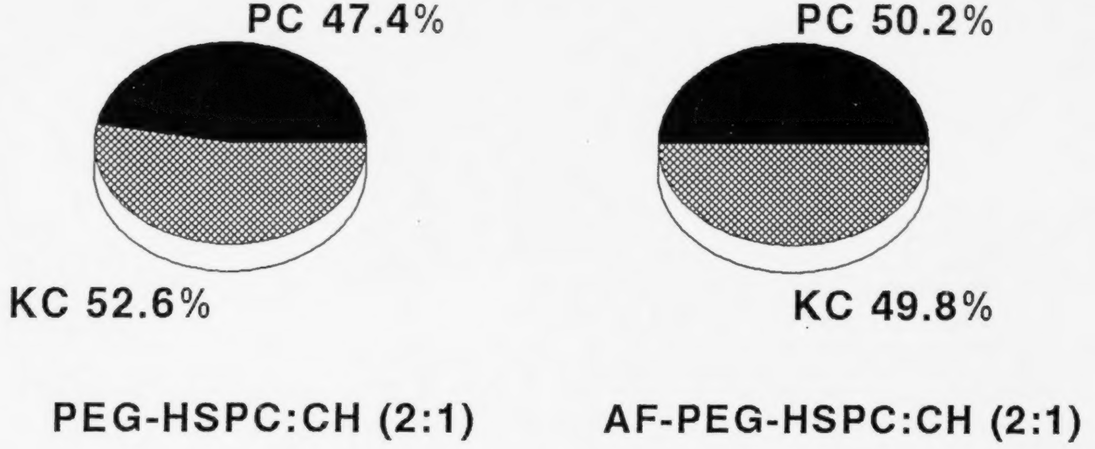


Table 11 Intrahepatic distribution of various liposomes in rats.

Four different compositions of liposomes labelled with ^{3}H-COE-lipid were injected into male Wag/Rij rats (n = 1-3), by the intravenous route (penis vein). Rats received 3 μmol of liposomal phospholipid in less than 1 ml of HEPES buffer saline (pH 7.4). At 24 hours after the injection, the animal was anaesthetized and sacrificed. Liver perfusion and isolation of parenchymal cells (PC) and Kupffer cells (KC) were performed as described in Materials and Methods. Liver distribution was expressed as the percentage of various liposomes recovered in a specific cell population (e.g. PC or KC) relative to the total liver uptake of that particular liposome type. Mean ± S.D. for HSPC:CH (n = 3).

Composition	Hours after injection	parenchymal cells (%)	Kupffer cells (%)
HSPC:CH (2:1)	24	33.5 ± 14.7	66.5 ± 14.7
PEG-HSPC:CH (2:1)	24	46.5, 48.3	53.5, 51.7
AF-PEG-HSPC:CH (2:1)	24	53.1, 47.3	46.9, 52.7
AF-HSPC:CH (2:1)	24	56.7	43.3

This indicated that the longer half life of PEG-liposomes, increased their access to parenchymal cells. However, conjugation of asialofetuin to PEG-liposomes (AF-PEG-HSPC:CH) did not significantly change their association with parenchymal cells (Fig. 16C, Table 11), even though the conjugation ratio of AF-PEG-liposomes (4.56×10^{-4} μmol AF/μmol PL, or 24 μg AF/μmol PL; Table 8B) was two-fold higher than that of AF-liposomes (2.28×10^{-4} μmol AF/μmol PL, or 12 μg AF/μmol PL; Table 8B). This indicated that incorporation of PEG into liposomes may result in the steric interference of PEG with the binding of asialofetuin to its receptor.

IV. DISCUSSION

(A) *Factors Involved in Receptor Recognition*

Liposomes associate with cells mainly by three different means, i.e., non-specific adhesion to the surface of the cell membrane, specific recognition of targeted liposomes by their receptors, e.g., surface glycoproteins or proteins, on cell membrane, and internalization resulting from either non-specific adhesion or specific recognition (Martin et al., 1990). Because the liposome uptake in this study is measured as the amount of the non-exchangeable/non-metabolizable ^{3}H-CHE label associating with 2.2.15 or HepG2 cells, the term "uptake" means a summation of the amount of liposomes associating with cells by non-specific adhesion, specific recognition (if there is any), and internalization.

(1) Influence of the Structure of ß-Galactose-receptor Ligands on the Recognition of Liposomes by 2.2.15 Cells

The results of comparative experiments showed that the hepatic uptake of AF-liposomes was superior to ASGM$_1$-liposomes, LAC-liposomes, and immunoliposomes against HBsAg (Fig. 2; 4 and Table 5). When considering the density of galactose residues on different liposomes, the benefit of using asialofetuin was even more obvious (Table 1). Asialofetuin resulted in a more than three-fold increase in uptake over plain liposomes by 2.2.15 cells as compared to lactosylceramide or asialo-GM$_1$, while the density of galactose residues on AF-liposome surface (1.56×10^{-4} μmol galactose residue/μmol PL) was only 1/30 of that on the surface of LAC- or ASGM$_1$-liposomes. Because lactosylceramide and asialo-GM$_1$ were incorporated into the lipid bilayers in a symmetrical manner, only approximately 50% of the lactosylceramide or asialo-GM$_1$, i.e., 0.05 μmol ligand/ μmol PL in this study, would be on the outer surface of the liposomes. The rest of the incorporated ligands were on the internal bilayer surface.

The increased uptake of AF-liposomes can be explained by a number of factors:

(a) length of the carbohydrate residues of various galactose-containing ligands

Asialo-GM$_1$ has a longer sugar chain (with 4 sugar residues) which results in its terminal galactose residues extending farther out from the liposomal surface where they may more readily bind to ß-galactose receptors on liver cells, as compared to LAC which has only two sugar residues (Fig. 17). This appears to result in somewhat increased uptake of ASGM$_1$-liposomes by 2.2.15 cells at low phospholipid concentrations (< 50 nmol/ml) (Fig. 5), as compared to LAC-liposomes under the same conditions (Fig. 3). However, this longer length of ASGM$_1$ does not dramatically increase the intake of ASGM$_1$-liposomes by liver cells, as compared to LAC-liposomes.

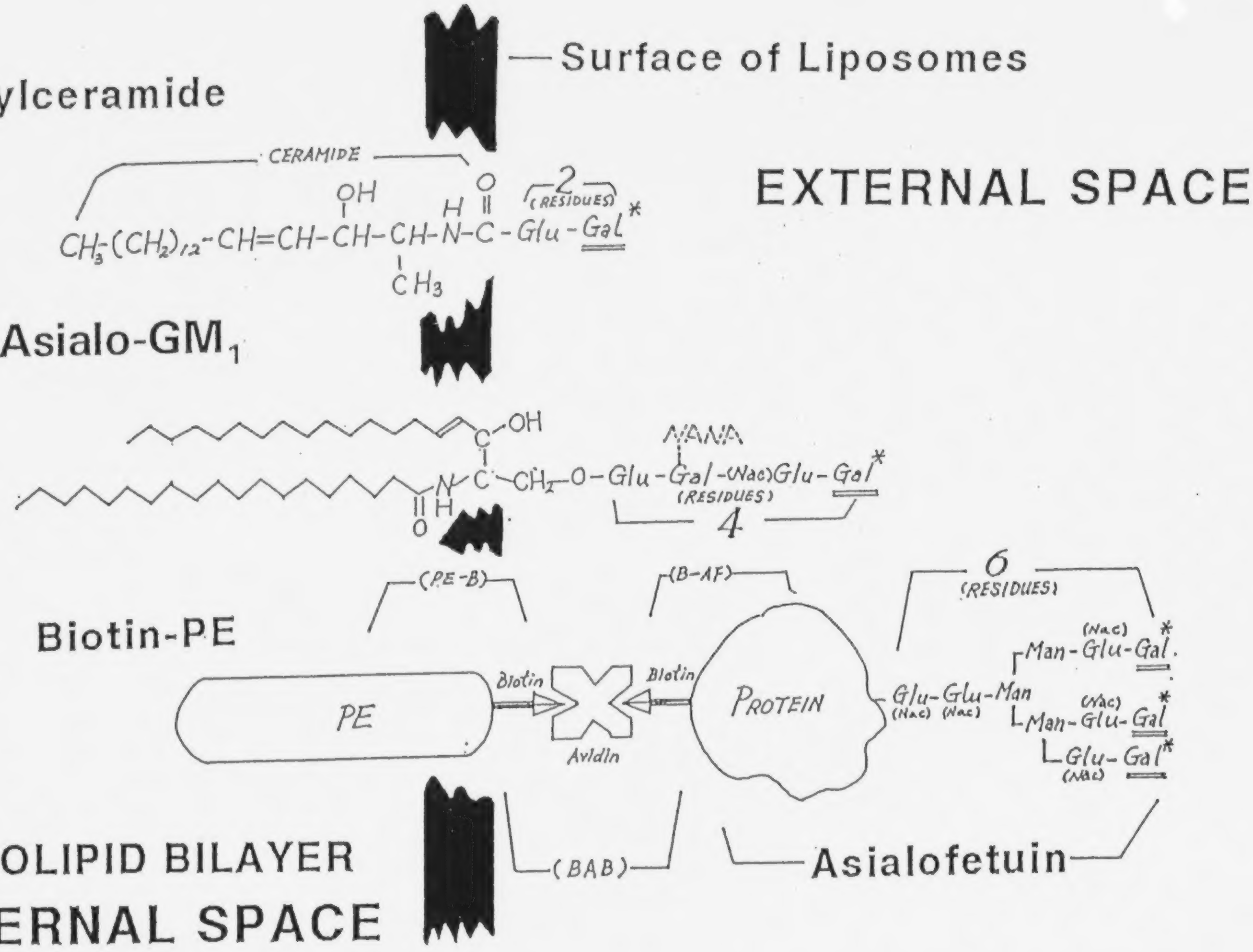
The advantage of a long sugar chain is more apparent in the case of AF-liposomes. Asialofetuin itself has 6 residues in each branch of its tri-antennary sugar chain (Fig. 17). That is four more sugar residues than lactosylceramide. The presence of the avidin protein in the coupling method used further lengthens the distance of the galactose residues from the liposomal surface. Thus, the terminal galactose residues of AF-liposomes are more exposed than those of LAC- or ASGM$_1$-liposomes. This allows AF-liposomes to be more easily recognized than LAC- or ASGM$_1$-liposomes (Fig. 17).

(b) multivalent binding to the galactose receptors

The tri-antennary structure of asialofetuin, with a terminal galactosyl residue on each branch, and its long sugar chain offer a better opportunity for multivalent binding, which is required for stable binding to ß-galactose receptors, and for triggering the internalization of receptors occupied by AF-liposomes (for review : Ashwell and Harford, 1982). As mentioned previously, a simultaneous binding at two receptor sites, 25-30 Å apart, is essential for the endocytosis of ligands bound to ß-galactose receptors (Baenziger and Maynard, 1980). This characteristic of multivalent binding contributes to the lowered affinity of asialotransferrin to ß-galactose receptors because its bi-antennary sugar chain is less capable of binding to two separate receptor sites than are other asialoglycoproteins with more branches on their sugar

Figure 17 Schematic diagram of ß-galactose receptor ligands.

This diagram shows the basic skeletons of lactosylceramide, asialo-GM$_1$ and asialofetuin. Their positions relative to the liposome surface and to each other, after insertion or conjugation, are also demonstrated. Diagram is not shown to scale. Glu, Glucose; Gal, Galactose; Glu(Nac), N-acetyl-D-glucosamine; NANA, Sialic acid (* This diagram shows the structure of asialo-GM$_1$. The position of the sialic acid group before desialylation is indicated with broken symbols); BAB, biotin-avidin-biotin; PE-B, biotin-PE; B-AF, biotin-asialofetuin.



Lactosylceramide

Surface of Liposomes

EXTERNAL SPACE

Asialo-GM$_1$

Biotin-PE

Asialofetuin

PHOSPHOLIPID BILAYER

INTERNAL SPACE

chains (Morell et al., 1971; Ashwell and Harford, 1982). The multivalent binding theory can also explain the critical density required for the recognition by ß-galactose receptors of liposomes containing asialoganglioside or lactosylcerebroside because the monovalent character of the ligands asialoganglioside or lactosylcerebroside would require high surface density for simultaneous binding to two or more receptor sites (Ghosh et al., 1980; Szoka and Mayhew, 1983).

The requirement for multivalent binding may also restrict the use of LAC- or ASGM$_1$-liposomes smaller than 80 nm. In a recent *in vivo* investigation, done by Scherphof and coworkers (1994), a very interesting phenomenon was observed. Small LAC-PEG-liposomes, i.e., LAC-PEG-egg PC:CH (2:1) liposomes (≈ 85 nm in diameter), had a significantly lower total liver uptake than did large LAC-PEG-liposomes (≈ 125 nm in diameter), while spleen uptake in both cases was similar.

A small liposome size may decrease simultaneous binding of individual galactose moieties on LAC-PEG-liposomes with ß-galactose receptors. Scherphof et al. have explained "... that a predominant role is played by the way in which the galactose moieties are exposed in both liposome types (small and large) and the degree of curvature of the vesicles plays an important role in this. On the one hand, strong curvature may cause the PEG chains on the liposomal surface to spread more widely apart, making the galactose moieties in between more accessible and leading to better recognition. On the other hand, however, strong curvature may impede the proper alignment of the galactose moieties for multivalent interaction with the receptors and thus cause a weaker liposome-receptor interaction. Presumably then, the alignment effect prevails over the exposure effect, causing a net reduction in receptor recognition by the smaller vesicles (85 nm)" (Scherphof et al., 1994). To conclude from above, there is a potential disadvantage for using LAC- or ASGM$_1$-liposomes to target hepatocytes, that is, their poor ability to participate in multivalent binding.

In comparison, asialofetuin has a triantennary sugar chain and an efficient structure for multivalent binding with ß-galactose receptors. This advantage, combined with the more exposed terminal galactosyl residues, overcomes the disadvantage of low galactosyl density on AF-liposomes and results in the higher *in vitro* uptake of AF-liposomes on both HepG2 and 2.2.15 cell line than is seen with LAC- or $ASGM_1$-liposomes.

The magnitude of ligand-liposomal uptake is not only determined by those properties of various ligands discussed above, but also by the dynamic collision between liposomes and surface receptors. The latter is mainly dependent on the concentration of liposomes (i.e., phospholipid concentration), the density of ß-galactose receptors on the cell membrane, the temperature, and the binding affinity of the ligand for its receptor. In our uptake studies, the cell number (interpreted as a function of the receptor number of that particular cell type) of each experimental sample and the temperature are controlled parameters, thus the dynamic collision is mainly determined by the phospholipid concentration. Generally, the presented data showed that liposomal uptake increased with the increase of phospholipid concentration. However, the suspected bi-phasic uptake (Fig. 2 and 4) of ligand-liposomes by 2.2.15 or HepG2 cells suggests a possible involvement of other mechanisms.

As already mentioned, the magnitude of ligand binding is determined by both the dynamic collision and the specific properties of different ligands. However, at low phospholipid concentrations (corresponding with the low odds of collision), the specific properties of various ligands become the major determinants. When the odds of collision are low, an effective collision, which results in a stable multivalent binding between a ligand and its receptor, becomes a more rare event. The chances of an effective collision resulting in receptor binding is more dependent on the properties of ligands, such as the length and the branches of their sugar chain, at low phospholipid concentrations. The difference between the degree of receptor binding of ligand-liposomes at low and high concentrations resulting in an apparently bi-phasic binding curve suggest that a critical concentration of phospholipid is required for effective binding, especially in the cases of LAC- and $ASGM_1$- liposomes.

Presented data suggest that at low phospholipid concentrations, $ASGM_1$-

liposomes and LAC-liposomes associated with 2.2.15 cells to different degrees because of the differences in the lengths of their sugar chains. In comparison, at high concentrations ($\geq$ 500 nmole PL/ml), this slight difference between the uptake of ASGM$_1$- and LAC-liposomes became non-significant, i.e., the advantage of the two residue longer sugar chain of ASGM$_1$-liposomes may be masked by the significantly increased dynamic collision rate at phospholipid concentrations over a critical point.

(c) down regulation of ß-galactose receptors due to HBV infection

Another observation from comparative experiments is that the higher uptake of liposomes, mediated by ß-galactose receptors, by HepG2 cells over that by 2.2.15 cells under the same conditions (Table 5). This can be explained by the down regulation of surface ß-galactose receptors on 2.2.15 cells because of the HBV infection, as reported by several other groups (for review: Meijer et al., 1992). Figure 1 also shows the difference between the uptake of plain liposomes by 2.2.15 and HepG2 cells, indicating that HBV infection has altered, in some way, other properties of hepatoma cells. These observations suggest that there may be a problem in targeting ß-galactose receptors on HBV-infected hepatocytes because the HBV-infected cells may have altered receptor characteristics (e.g. less surface or exposed receptors, prolonged recycling time of receptors or changed receptor conformation) depending on the state of the disease. Thus, it should be kept in mind that the concept of targeting surface receptors may have unforseen limitations in the case of virally-infected cells (Ho et al., 1987a,b).

(2) Problems with HBsAg as a Receptor for Liposomal Targeting to 2.2.15 Cells

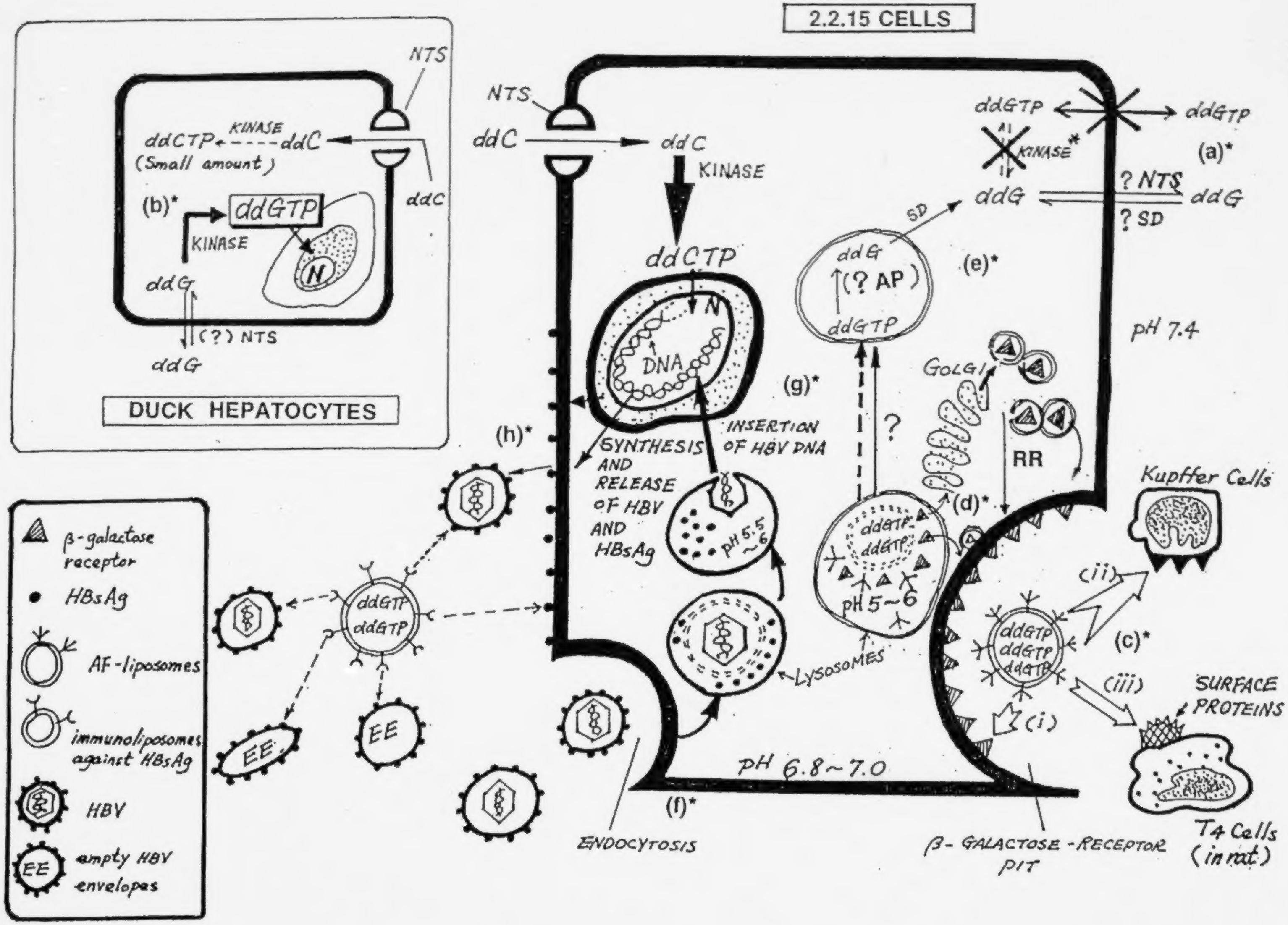
Targeting immunoliposomes to 2.2.15 cells was not as effective as expected (Fig. 10). In these experiments, conjugation of mAb against HBsAg (*ad + ay* type) to liposomes resulted in only a two-fold increase of uptake over plain liposomes and was 50% lowered than that of AF-liposomes (Table 5). Because the conjugation ratio of mAb-against-HBsAg obtained in this study is relatively low (13 μg mAb/μmol PL), due to the low initial concentration of purchased mAb, this may have resulted in the low levels of recognition of mAb-liposomes by surface HBsAg on 2.2.15 cells. Several additional factors may also contribute to this result.

First, the mAb used in this study is a broad type which recognizes both *ad* and *ay* subtypes of HBV. However, the 2.2.15 cell line was developed from a HepG2 cell line transfected with HBV mainly from North American patients. A possible reason for the low uptake of mAb-liposomes is therefore the lack of the specific *ad* subtype, which is dominant in Asian people, on the 2.2.15 cells (Acs et al., 1987; Sells et al., 1987; 1988). Another possible factor for poor immuno-targeting is that the expression of HBsAg on the surface of 2.2.15 cells is time-course dependent, i.e., high density of surface HBsAg is present only at a particular time corresponding to the replication cycle of HBV. At the end of the replication cycle, large amounts of replicated HBV-DNA are released from the nuclei. After coating with the viral envelopes, HBV are exocytosed. At the same time, empty viral envelopes with surface HBsAg are also released from cells and large amounts of HBsAg are found on the cell membranes (Lycke and Norrby, 1983). During the resting period, HBsAg is barely replicated and its density on the cell surface is relatively low. Thus, it is anticipated that the binding of immunoliposomes against HBsAg with 2.2.15 cells will be low during this resting period. Since we do not know the exact time when the HBsAg is released in a large scale, optimization of the uptake of mAb-liposomes becomes difficult.

If the timing of high surface HBsAg density could be determined and treatment with immunoliposomes given at that time, an additional problem could arise. The empty viral envelopes released coincidentally into the media may compete to a significant degree with the specific binding between mAb-liposomes and HBsAg on the 2.2.15 cell surface (Fig. 18) (Sells et al., 1987; 1988). This is a major obstacle for the clinical use of mAb-liposomes against HBsAg, because high levels of plasma HBsAg from circulating empty envelopes is always detected in both acute HBV

Figure 18 **Schematic diagram of the interactions between AF-liposomes, mAb-'posomes and 2.2.15 cells.**

The kinases which phosphorylate pyrimidine ddNs into pyrimidine ddNTPs appear to be present in higher levels in 2.2.15 cells, than in duck hepatocytes. By contrast, the specific kinases responsible for the formation of purine ddNTPs (e.g. ddGTP) are less active or have lower intracellular concentrations in 2.215 cells than in duck hepatocytes (Lee et al., 1989; Kitos, 1994). A specific nucleoside transport system (NTS) for purine ddNs has not yet been identified on the membrane of 2.2.15 cells. It is not clear whether ddG (or other purine ddNs, such as ddA or ddl) are transported into the 2.2.15 cells by simple diffusion (SD) and/or by a more sophisticated mechanism, such as the NTS. 2.2.15 cells do not effectively convert ddG into ddGTP because of the extremely low amount of purine ddN kinases in this particular cell line [(a)* in diagram]. By contrast, duck hepatocytes convert ddG into ddGTP efficiently and the produced ddGTP successfully inhibits the DHBV replication [(b)*] (Lee et al., 1989; Kitos,1994). AF-liposomes may be recognized by i) ß-galactose receptors on 2.2.15 and hepatic parenchymal cells, ii) galactose particle receptors on Kupffer cells, and iii) a group of specific surface proteins on T4 cells, in rats [(c)*]. Our studies show that AF-liposomes bind to 2.2.15 cells via the surface ß-galactose receptors resulting in a significantly increased uptake over plain liposomes. Additionally, AF-liposomes are good carriers for ddGTP. After binding, AF-liposomes are internalized by 2.2.15 cells likely by transport into lysosomes. In lysosomes, liposomes and their contents are hydrolysed and the ß-galactose receptors are recycled to the cell surface (RR = receptor recycle) [(d)]*. The acid phosphatase (AP) in the lysosomes may be responsible for inactivation of ddGTP, converting it back to ddG. Because of the inactivation of ddGTP and the lack of Kinases catalyzing the formation of ddGTP, AF-liposomes containing ddGTP show no inhibition of HBV replication in 2.2.15 cells [(e)*]. HBV is taken into hepatocytes via endocytosis [(f)*]. HBV DNA is inserted into the nuclei (N) and fused with cellular DNA, resulting in the synthesis and release of HBV and HBV related antigens (i.e., HBsAg, HBcAg and HBeAg)[(g)*]. HBsAg is expressed on the cell membrane at the later stage of HBV replication [(h)*]. Because anti-HBsAg-immunoliposomes bind to the released virus and their empty envelopes (EE), as well as the surface HBsAg on the membrane, targeting of these immunoliposomes to 2.2.15 cells is interfered with [(h)*].


2.2.15 CELLS
DUCK HEPATOCYTES
NTS
ddCTP ← KINASE - - - ddC
(Small amount)
ddC
(b)*
ddGTP
KINASE
N
ddG
(?) NTS
ddG
NTS
ddC
ddC
KINASE
ddCTP
N
DNA
(g)*
INSERTION OF HBV DNA
SYNTHESIS AND RELEASE OF HBV AND HBsAg
pH 5.5 ~ 6
Lysosomes
(h)*
ddGTP
ddGTP
ddG
(? AP)
ddGTP
SD
(e)*
?
pH 5~6
ddGTP
ddGTP
(d)*
GOLGI
RR
ddGTP
ddGTP
KINASE*
(a)*
ddG
? NTS
? SD
ddG
pH 7.4
Kupffer Cells
(ii)
(c)*
ddGTP
ddGTP
ddGTP
(iii)
(i)
SURFACE PROTEINS
T4 Cells (in rat)
pH 6.8~7.0
(f)*
ENDOCYTOSIS
β- GALACTOSE -RECEPTOR PIT
EE
EE
β-galactose receptor
HBsAg
AF-liposomes
immunoliposomes against HBsAg
HBV
EE
empty HBV envelopes

patients and in syndrome-free carriers (Chen, 1993). It is likely that, after injection, mAb-liposomes against HBsAg will bind mainly to the empty envelopes competing for binding to HBsAg located on the infected hepatocytes. In light of the anticipated difficulties in the use of mAb-liposomes against HBsAg and the relative success of AF-liposomes, we decided to focus our research on the ß-galactose receptor-mediated uptake of liposomes by hepatocytes.

(B) *Optimization of Encapsulation Efficiency*

We observed that efficiency of drug entrapment varied with experimental conditions. High entrapment efficiency is desirable so that antiviral drug is not wasted. Two approaches may be used to improve the efficiency of drug capture. The initial concentrations of phospholipids and of ddGTP used to rehydrate the phospholipid film are two determining factors which can influence the efficiency of entrapment. By utilizing either higher concentrations of ddGTP and/or phospholipid, the efficiency might be improved. However, when the phospholipid concentration was low, simply elevating the ddGTP concentration may not significantly improve the efficiency of entrapment, because the liposome entrapped volume relative to the bulk solution will be low. A more reasonable way to increase efficiency of entrapment would be to elevate the initial concentration of phospholipids. Indeed, Table 3B shows that the higher the initial concentration of both phospholipid and ddGTP, the higher the entrapment efficiency. Increasing the initial phospholipid concentration to 100 μmol PL/ml greatly improved the efficiency of entrapment. However, increasing only the initial concentration of ddGTP by four times had less of an effect on entrapment efficiency (Table 3B).

The encapsulation and stability of ddNTPs in liposomes has been investigated by several groups (Betageri, 1993; Szebeni et al., 1990). It has been demonstrated that ddNTPs are good candidates for liposomal encapsulation and are very stable after encapsulation, if stored at 4°C. The presence of cholesterol in liposomes (with a molar ratio of PL:CH, 2:1) greatly increases the stability of ddNTPs in liposomes

(Betageri, 1993; Szebeni et al., 1990). The results presented in this thesis further support the concept that incorporating a suitable amount of cholesterol into liposomes may increase the stability of contents entrapped in liposomes. It is clear that the basic liposomal composition (HSPC:CH 2:1) in our investigation prevents the leakage of ddGTP from liposomes and offers a satisfactory level of encapsulation (Fig. 19). The increased rigidity conferred to liposomes by HSPC, whose fatty acid chains are saturated (indicating a higher phase transition temperature, 55-60°C), may also contribute to this favourable encapsulation and decreased leakage. The liquid-crystalline phase transition of phospholipid bilayers is temperature dependent, i.e., the transition occurs when the environmental temperature is over the phase transition temperature (T_c) of the particular phospholipid type in the liposomes. In general, the longer the length and higher the saturation degree of the fatty acid chain, the higher the T_c. Transition of phospholipid bilayers from solid (gel) phase into fluid (crystal) phase leads to increased fluidity and permeablilty of liposomal bilayers (Martin et al., 1990). HSPC, a rigid phospholipid, has a T_c of about 55-60°C, thus the liposomes will be below their transition temperature at physiological temperature, and the rate of leakage of ddGTP from liposomes containing HSPC would be low.

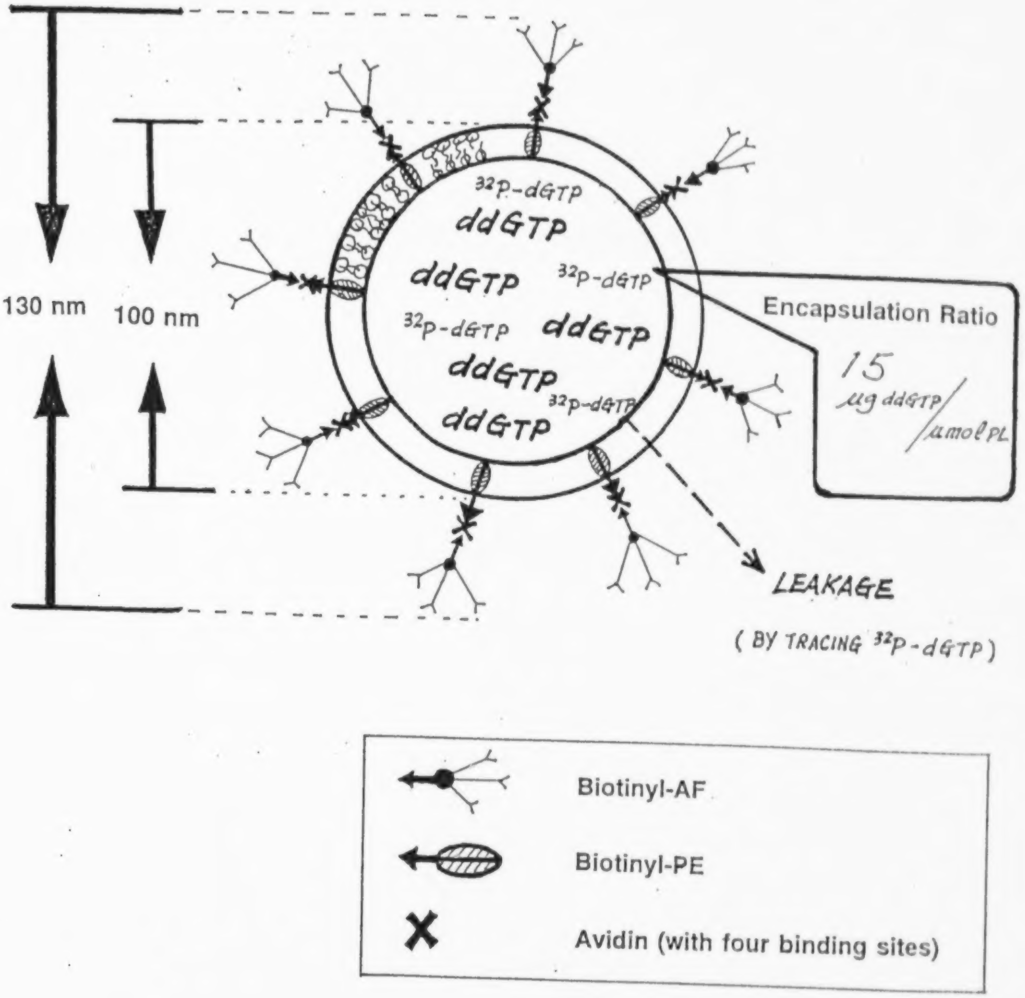
In this study, leakage rate was determined by measuring the radio-label, ^{32}P-dGTP. Since dGTP has an -OH group on the 3′-position of ribose, dGTP is somewhat more hydrophilic than ddGTP. This indicates that ^{32}P-dGTP tends to leak out from liposomes more slowly than does intact ddGTP. However, the bulky triphosphate group in the dGTP or ddGTP is the predominant group determining the polarity of the whole molecule and makes the difference in polarity between dGTP and ddGTP, resulting from the -OH group in dGTP, become insignificant. In the mixture of ddGTP with ^{32}P-dGTP for studying the leakage and entrapment ratio, the interaction between relatively little amount of ^{32}P-dGTP and bulk of ddGTP would lead to the even less significant difference between ^{32}P-dGTP and ddGTP. Thus, it is important to point out that using ^{32}P-dGTP to monitor the leakage of ddGTP from liposomes may lead to a considerable but not significant limitation.

(C) *The Fate of Liposomes after Uptake by Cells*

The internalization of ligand bound ß-galactose receptors was demonstrated by

Figure.19 Schematic diagram of the encapsulation and leakage of ddGTP from AF-liposomes.

ddGTP, labelled with ^{32}P-dGTP, was entrapped into liposomes during the rehydration step of liposome formation as described in Materials and Methods. By measuring the ^{32}P-radioactivity encapsulated in the liposomes, the encapsulation ratio of ddGTP to phospholipid was determined. It was found that every one μmole of phospholipid (in this case HSPC) captured approximately 15 μg of ddGTP. By monitoring the radioactivity of ^{32}P, the rate of leakage of ddGTP from liposomes was monitored. It was found that storage at 4°C prevented the leakage of ddGTP. The half life of ddGTP leakage at 37°C from AF-liposomes in buffer was about six days.


130 nm
100 nm
^{32}P-dGTP
ddGTP
ddGTP
^{32}P-dGTP
^{32}P-dGTP
ddGTP
ddGTP
^{32}P-dGTP
ddGTP
Encapsulation Ratio
15 µg ddGTP/µmol PL
LEAKAGE
(BY TRACING ^{32}P-dGTP)
Biotinyl-AF
Biotinyl-PE
Avidin (with four binding sites)

using a pH-sensitive fluorescent probe. Other attempts at targeting ligand-incorporated liposomes to ß-galactose receptors, the internalization of ligand-liposomes was also demonstrated (Ghosh and Bachhawat, 1980; Dasgupta and Bachhawat, 1985). In a recent paper (Tsuchiya et al., 1991), it was shown that approximately one third of liposomes containing asialofetuin (using a detergent-removal method which inserts asialofetuin into liposomes non-specifically) associated with HepG2 cells were internalized. By using AF-liposomes containing HPTS, we provided results showing that liposomes conjugated to asialofetuin were internalized by 2.2.15 cells.

After incubation with 2.2.15 cells for two hours, it was found that HPTS was in an environment of pH 6, clearly indicating that AF-liposomes were taken into 2.2.15 cells and possibly localized in the lysosomes. After incubation for twenty four hours, the internalized AF-liposomes with loaded HPTS appeared to be transported into an organelle with higher pH value (6.9), likely the Golgi-apparatus (Fig. 18 and 20).

(D) *Factors Affecting the Antiviral Efficacy of AF-liposomes Containing ddGTP*

Several factors will influence the antiviral activity of AF-liposomes containing entrapped ddGTP, as discussed below.

(1) Dose of ddGTP

From the data for uptake of AF-liposomes by 2.2.15 cells and the entrapment ratio, can be calculated that incubation of AF-liposomes (1000 nmol of PL/ml) with an entrapment ratio of 15 μg ddGTP/μmol PL (= 15000 pg ddGTP/nmol PL) can lead to the association of about 45 pmol of ddGTP with one million cells in one hour[1], and approximately one third of this amount of ddGTP would be internalized, as suggested by Tsuchiya and coworkers (1991). In a recent study (Kitos, 1994), it was found that incubation 4 μM of ddG with 2.2.15 cells for twenty six hours resulted in less than 1 pmol of ddGTP in one million cells. The dosage given in our experiments is expected to introduce about 10-15 pmol of ddGTP into one million cells (i.e., at least ten times the ddGTP obtained by incubation of 4 μM ddG with 2.2.15 cells for twenty-six hours). Based on the assumption of 45 pmol ddGTP associating with one million 2.2.15 cells,

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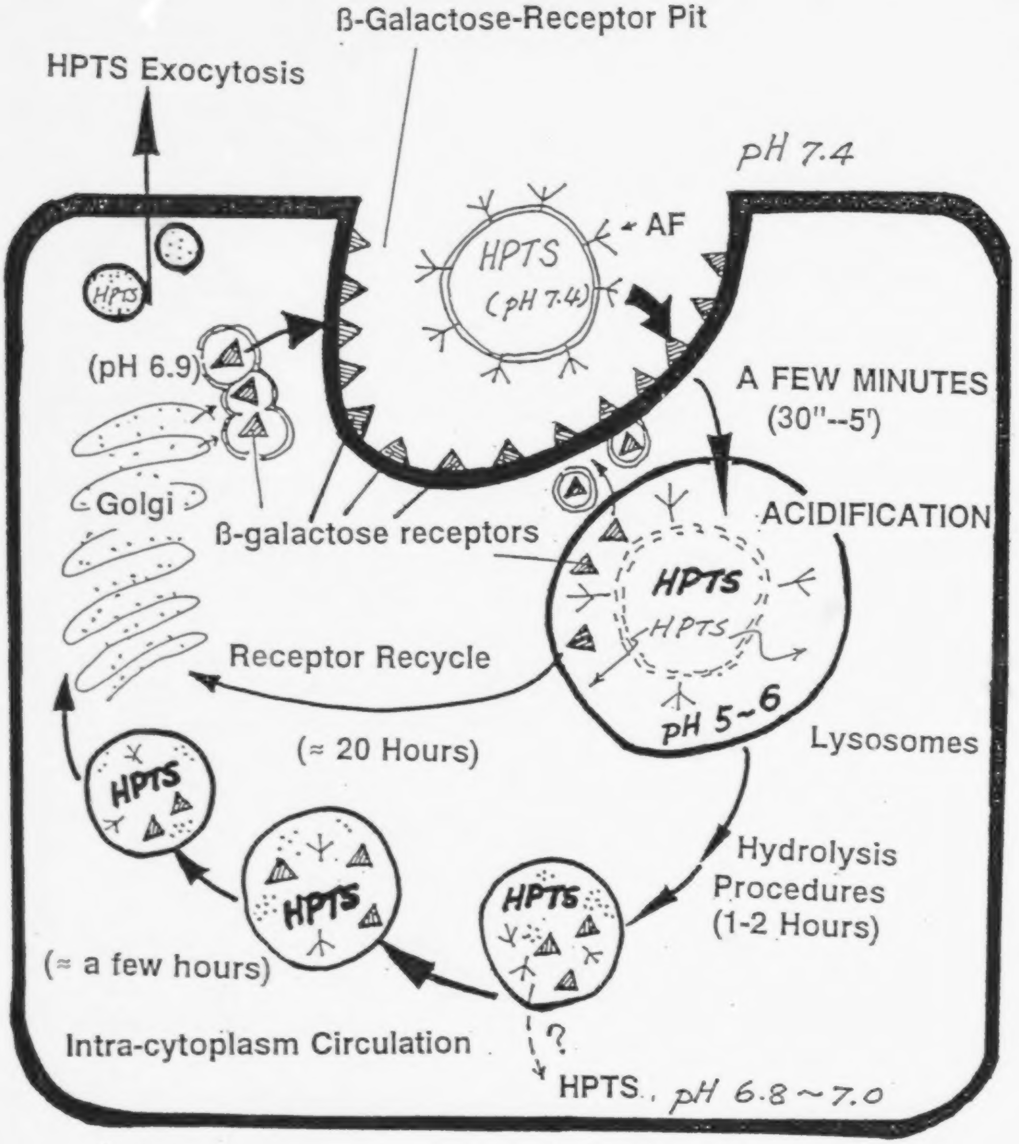
[1] Uptake of AF-liposomes by 2.2.15 cells, after one-hour incubation at 37°C
= 6 nmol PL/4x10^6 cells/hr (Table 5)
= 1.5 nmol PL/10^6 cells/hr
= 1.5x15000 (= 22500) pg ddGTP/10^6 cells/hr
≈ 45 pmol ddGTP/10^6 cells/hr

Figure 20 Schematic diagram of the internalization of AF-liposomes containing HPTS by 2.2.15 cells.

To investigate the internalization of liposomes, HPTS (1-hydroxypyrene-3,6,8-trisulfonic acid) was entrapped into liposomes as described in Materials and Methods. HPTS is a pH-sensitive fluorescent material and is not permeable from liposomes after encapsulation. HPTS emits fluorescence at different wavelength corresponding with the change in pH of its environment. Thus, by recording the emission profiles of HPTS entrapped in liposomes, the pH value of the environment to which liposomes are exposed can be monitored. After incubation of AF-liposomes containing HPTS with 2.2.15 cells at 37°C for 2 hours, the environmental pH was in the range of 5.5 to 6.0 agreeing with the pH value inside of endosomes/lysosomes. This indicated that AF-liposomes were internalized and may have been transported into endosomes/lysosomes after two-hour incubation. After incubation for 24 hours, HPTS was transported to an organelle with a pH value of 6.9, likely the Golgi apparatus.


ß-Galactose-Receptor Pit
HPTS Exocytosis
pH 7.4
HPTS
HPTS
(pH 7.4)
AF
(pH 6.9)
A FEW MINUTES
(30''--5')
Golgi
ß-galactose receptors
ACIDIFICATION
HPTS
HPTS
Receptor Recycle
pH 5~6
(≈ 20 Hours)
Lysosomes
HPTS
HPTS
HPTS
Hydrolysis
Procedures
(1-2 Hours)
(≈ a few hours)
Intra-cytoplasm Circulation
?
HPTS , pH 6.8 ~ 7.0

we expected that a fourteen-day protocol in which 1000 nmol PL/ml of AF-liposomes containing 30 μM[2] liposomal ddGTP, given every other day, should deliver a total amount of 70-105 pmol of ddGTP into one million cells in fourteen days and effectively inhibit HBV replication in 2.2.15 cells. However, HBV replication was not inhibited by this treatment.

(2) Possible Degradation of ddGTP in Lysosomes

The most likely explanation for the failure of ddGTP in AF-liposomes to inhibit viral replication is that ddGTP is hydrolysed into ddG by nucleoside phosphatase in lysosomes and cannot be efficiently converted back to ddGTP because of the lack in 2.2.15 cells of the specific kinases for converting ddG to ddGTP (Fig. 18).

Receptor-mediated internalization of liposomes by cells has been widely studied. Increasing evidence supports the view that receptor-targeted liposomes will be internalized into lysosomes where hydrolysis of phospholipids and other degradation takes place (for review see Meijer et al., 1992; Meijer and Ziegler, 1993). The results of our internalization experiments with liposomal HPTS also suggested that the most likely location of internalized AF-liposomes was inside hepatocyte lysosomes, after two hour incubation at 37°C. Lysosomes are well known as the decomposition factory for endocytosed materials and many lysosomal enzymes have been recognized (Barrett, 1984), including enzymes which degrade phospholipids. The so-called "acid phosphatases" (including a nucleoside triphosphatase) is a group of phosphatases responsible for degrading nucleotides into nucleosides which can penetrate the lysosomal membrane, and was first observed by Arsenis and coworkers in 1970 (Arsenis et al., 1970; Burton et al., 1975 and Lloyd and Forster, 1986). It is very possible that ddGTP, encapsulated in AF-liposomes, is de-phosphorylated by the acid phosphatase in lysosomes, and free ddG is released into the cytoplasm by simple diffusion (Fig. 18). In addition, even if there was no acid phosphatase in lysosomes, the acidification in lysosomes (Yamashiro et al., 1983) would strongly destabilize ddGTP and the phosphate groups would be eventually hydrolysed, one by one. As mentioned previously, it is suspected that 2.2.15 cells lack the ability to convert purine ddNs into their active form, ddNTPs (Lee et al., 1989; Kitos, 1994). Thus, the released ddG may not be converted back to ddGTP by 2.2.15 cells. This may defeat our attempts to use AF-liposomes containing ddGTP to inhibit HBV replication (Fig. 18).

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[2] 1000 nmol PL/ml = 15 μg ddGTP/ml = 30 μM ddGTP

(3) Possible Means of Preventing ddGTP Degradation

The degradation of ddNTPs in lysosomes may be a major disadvantage in the application of liposomal ddNTPs (Szebeni et al., 1990 and Meijer et al., 1992). To prevent the degradation of ddNTPs, several possibilities could be considered. Inhibiting the activity of acid phosphatase is the most straight forward method. However, this could also result in the eventual exocytosis of ddGTP by 2.2.15 cells because ddGTP may not penetrate lysosomal membranes.

Using pH-dependent liposomes, which fuse with physiological membranes at low pH (Yatvin et al., 1987), such as in lysosomes, may resolve part of the problem. Fusion of the lysosomal membrane with pH-dependent liposomes containing ddGTP may deliver some ddGTP into the cytoplasm before degradation but it is very difficult to determine the delivery efficiency of this method (Meijer et al., 1992).

Another possible approach is virosomes. Virosomes are composed of viral envelopes with their DNA or RNA cores removed by detergent and sequential centrifugation. They fuse with physiological membranes spontaneously (Wilschut et al., 1991, Schoen et al., 1994, Bron et al., 1993 and Bentz, 1993). Enveloped viruses, such as the influenza virus, utilize a membrane fusion strategy to deposit their genome into the cytoplasm of the host cell. Infectious cellular entry of enveloped viruses can occur either by fusion of the viral membrane directly with the cell membrane or by fusion from within the endosomes after uptake of intact virions through receptor-mediated endocytosis (Bron et al., 1993; Wahlberg et al., 1992). Using virosomes to deliver foreign substances to cells has been shown to be successful (Schoen et al., 1994). Thus, applying virosomes containing ddGTP may prevent lysosomal degradation and deliver ddGTP into cytoplasm of 2.2.15 cells directly.

However, it is difficult to target either pH-liposomes or virosomes because incorporation of receptor ligands may prevent their fusion with physiological membranes, in one way or another. This will require further study (Huang et al., 1987).

(4) Obstacles to Clinical Application of AF-liposomes

It appears that AF-liposomes are better recognized by parenchymal cells *in vivo* than are plain liposomes, because the uptake of AF-liposomes by parenchymal cells is significantly higher than that of plain liposomes by hepatocytes (Fig. 16A).

However, the increase in uptake of AF-liposomes by hepatocytes *in vivo* over plain liposomes was only modest. Additionally, the presence on liposomes of asialofetuin did not significantly increase the total liver uptake (Table 10). These results indicate that AF-liposomes may not be recognized *in vivo* only by ß-galactose receptors on hepatocytes, but also by other receptors recognizing AF-liposomes *in vivo*. For example, it was found that galactose-particle receptors located on Kupffer cells take up all galactosyl particles with a diameter larger than 10 nm and the uptake of galactosyl particles by these receptors depends on the density of their surface galactose residues (Roos et al., 1985, Meijer et al., 1992). Also, in a rat model, a group of receptors recognizing bi- and tri-antennary glycoproteins was also found on the surface of leucocytes, i.e., T_4 cells (Bezouska et al., 1985). This indicates that AF-liposomes may be recognized by at least three different kinds of receptors in rats (Fig. 18). These could contribute to a decrease in the specific uptake of AF-liposomes by hepatocytes (Fig. 18).

(5) Benefits and Disadvantages of Liposomes Containing Polyethylene Glycol

The addition of PEG into HSPC:CH liposomes resulted in a slightly longer circulation half-life (22-26 hours) in Wag/Rij rats, as compared to the same liposomes without PEG (18-19 hours). The long circulation half-life of the liposomes lacking PEG may be partly explained by the increased rigidity of our liposomes. In a previous study done in Wag/Rij rats (Scherphof et al., 1994), it was found that conventional liposomes containing fluid phospholipids, i.e., egg phosphatidylcholine (egg PC), instead of HSPC , had a $T_{1/2}$ of 9.8 hours. This is almost two-fold shorter than the half-life of HSPC:CH liposomes in this study, for liposomes of comparable diameter. However, addition of PEG to the fluid liposomes resulted in a $T_{1/2}$ of 24 hours, very similar to the $T_{1/2}$ for our PEG-HSPC:CH liposomes. The likely explanation for this observation is that the rigid (high phase-transition) phospholipid, e.g., HSPC, increases the rigidity of liposomes, compared to liposomes composed of egg PC. This increased rigidity contributes to longer circulation times of liposomes containing HSPC, because the rigid phospholipids tend to decrease opsonization of liposomes by plasma proteins (Allen and Everst, 1983; for review see Allen, 1994a).

In the study done by Scherphof and coworkers (1994), LAC-PEG-liposomes (LAC-egg PC:CH:PEG-DSPE, diameter (d) = 86 nm), and those lacking LAC (d = 85 nm), had circulation half-lives of 19 and 24 hours, respectively. These data are comparable to AF-PEG-liposomes in this study ($T_{1/2}$ of 22 hours; d = 85 nm) and PEG-liposomes lacking AF ($T_{1/2}$ of 26 hours; d = 85 nm), supporting a previous observation that " ... Although many preparations (of PEG-containing liposome formulations) are currently made with rigid DSPC or HSPC in combination with cholesterol, **equally** long circulation half-lives can be achieved when egg PC is substituted for the rigid phospholipids." (Allen, 1994a).

These comparisons indicate that addition of PEG into small rigid liposomes, e.g., HSPC:CH liposomes in our study, can only slightly increase the circulating time of rigid liposomes. In comparison, addition of PEG into small liposomes without rigid phospholipids, e.g., egg PC:CH liposomes, can substantially increase their lifespan in the circulatory system. To conclude from above, incorporation of PEG, which increases the hydrophilicity of the liposomal surface, results in a decreased rate and extent of the uptake of liposomes by the MPS (Fig. 21), and a prolonged half-life, which is independent of lipid phase-transition. This is opposite to what is seen in liposomes containing GM_1. Their long half-life had been shown to be dependent on phospholipid phase-transition, i.e., high phase-transition lipid is necessary for the long half-lives of liposomes containing GM_1 (see introduction; Allen and Chonn, 1987; Allen et al., 1994a).

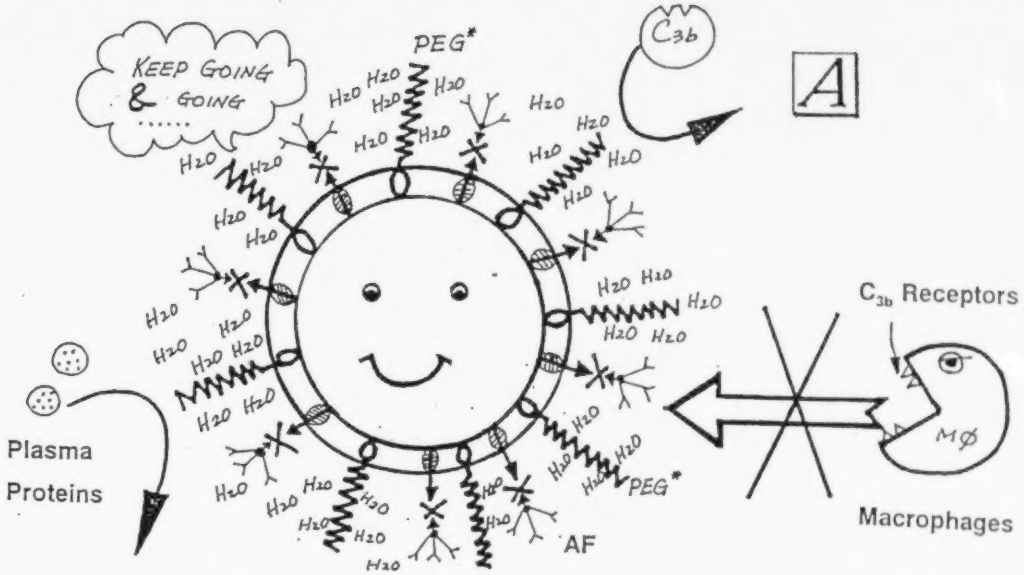
The small size of liposomes in the *in vivo* studies may also contribute to the relatively long half-lives of AF-HSPC:CH liposomes and those lacking AF. In general, smaller liposomes are removed from circulation more slowly than large liposomes (Allen et al., 1989; Allen and Everst, 1983; Allen et al, 1991b; for review see Allen, 1994a). It was found that the circulation half-lives in mice of PEG-containing liposomes with average diameters of 90-200 nm were significantly longer than those of liposomes larger than 300-400 nm in diameter (Kibanov and Huang, 1992). The relationship between decreased liposome size and prolonged liposome lifespan in the circulatory system may be a specific example of the general observation that clearance of particulate matter is proportional to particle diameter, i.e., the rate of clearance increases with increasing particle diameters (Allen, 1994a). The organ uptake of liposomes is also size-dependent. Long-circulating liposomes (containing PEG or GM_1) larger than 300 nm in diameter were mainly accumulated in spleen (Klibanov and Huang, 1992) and those of 90- 200 nm were mainly accumulated in the Kupffer cells in liver (Roerdink et al., 1981). Long-circulating liposomes with even smaller size (90-50 nm) had increased accumulation in liver, as compared to liposomes of 90-200 nm in diameter (Allen et al., 1989; Kibanov and Huang, 1992), because liposomes smaller than 90 nm more easily cross the fenestrated sinusoids (100-150 nm in diameter, Meijer et al., 1992; Meijer and Ziegler, 1993) in liver and gain access to liver parenchymal cells (hepatocytes) (Allen et al., 1989; Klibanov and Huang, 1992). The liposomes used in our *in vivo* studies were all in a size range of 80-90 nm in diameter (Table 8A). As a result from their very small size, the liposomes in this studies should have longer circulation half-lives and could access to parenchymal cells effectively.

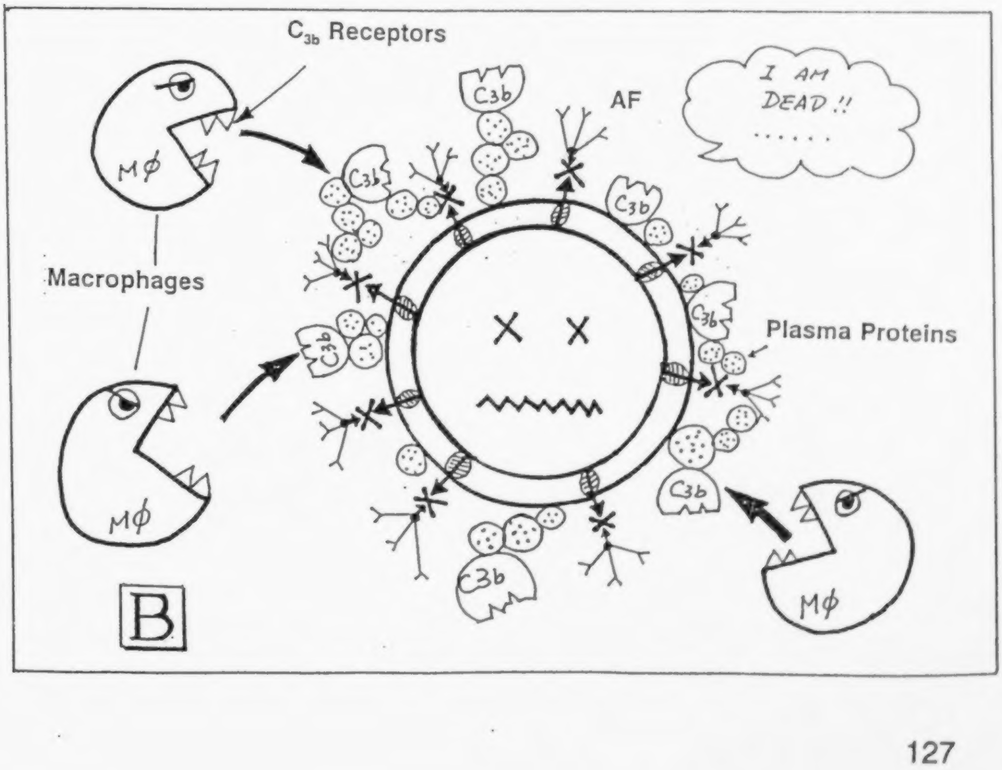
The dose of injected HSPC:CH liposomes or those with AF, in this study, may also result in the slight difference in the circulation half-lives between liposomes with and without PEG. Liposomes containing PEG-lipid or GM_1 have very different pharmacokinetics than do liposomes lacking PEG-lipid or GM_1. It was found that liposomes lacking PEG-lipid or GM_1 show saturable (Michaelis-Menton) pharmacokinetics, while liposomes containing PEG-lipid or GM_1 show nonsaturable (first-order, log-linear) kinetics (Allen and Hansen, 1991). The saturable kinetics of liposomes lacking PEG-lipid or GM_1, e.g., HSPC:CH and AF-HSPC:CH liposomes in this study, results in the circulation half-lives increasing with increasing injected doses of liposomes. Liposomes lacking PEG-lipid or GM_1 at low injected doses were rapidly removed from circulation following intravenous injection, with an initial rapid phase of removal followed by one or more slow phases of removal (Allen and Hansen, 1991). At low doses, liposomes containing rigid phospholipid, e.g., HSPC, in this study, were also removed from circulation in a multiphasic removal process, but with slower initial half-lives (Allen and Everst, 1983). As the dosage of injected liposomes lacking PEG-lipid or GM_1 increases, "...the processes contributing to rapid uptake of liposomes, i.e., recognition, binding and internalization, become saturated, and the circulation half-lives of liposomes increases as the slower phases of liposome removal become dominant. Processes that contribute to the slower phases of liposome removal from circulation likely include uptake into other tissues and turnover of binding sites, lipid metabolism, etc." (Allen, 1993). At a very high injected dose of fluid liposomes without PEG or GM_1 (10 μmol/mouse), clearance from blood of such liposomes following intravenous administration almost showed a log-linear pharmacokinetics in mice (Allen and Hansen, 1991). The evidence of saturation of the rapid phase of uptake in mice can be seen at doses as low as 2 μmol PL/kg of egg PC:CH liposomes (100 nm in diameter). In contrast, liposomes containing PEG-lipid or GM_1, e.g., PEG-HSPC:CH liposomes with or without AF in this study, were removed from circulation following intravenous injection in a primarily log-linear (which is dose-independent) process with initial half-lives of 12 to 24 hours (Fig. 15 C, D), depending on liposome size (Allen et al., 1989). The injected dose of liposomes in this study was 3 μmol PL/rat. Because the average weight of a rat used in this study is 250 g, the injected dose of liposomes was approximately 12 μmol PL/kg. Under this injected dose (12 μmol/kg), it was possible that the removal of HSPC:CH liposomes (with or without AF) was experiencing some degree of saturation of the MPS uptake (or slower removal processes), leading to longer circulation half-lives of such liposomes and the likely log-linear pharmacokinetics of their clearance from blood (Fig. 15 A, B). As a result, the difference in half-lives between liposomes with and without PEG was not very large as seen in our *in vivo* study.

The data also showed that the presence of PEG in liposomes in the absence of AF or other ligands could slightly increase the uptake of liposomes into parenchymal cells relative to plain liposomes (Fig. 16B), indicating that the longer circulation time of PEG-liposomes could result in greater penetration of the liver sinusoids by the liposomes, as compared to liposomes lacking PEG, leading to increased liposome uptake by hepatocytes (Fig. 21). On the other hand, the difference in uptake by hepatocytes between AF-PEG-liposomes and plain PEG-liposomes was not significant (Fig. 16C), i.e., the presence of asialofetuin in PEG-liposomes did not increase parenchymal uptake. As mentioned previously, the average diameter of PEG-HSPC:CH liposomes was not significantly different from the diameter measured after they were conjugated to AF (Table 8A), indicating that the AF-biotin-avidin complex might not be completely exposed on the surface of liposomes containing PEG. Thus, a possible explanation is that the long PEG polymers on the liposome surface may either mask the liposomal asialofetuin or interfere with the multivalent binding between asialofetuin and ß-galactose receptors, because the total length of an AF-biotin-avidin complex is not much longer than a PEG chain. Either way, the recognition or non-recognition of asialofetuin by hepatic ß-galactose receptors did not result in an increase in receptor-mediated uptake by hepatocytes *in vivo*.

Figure 21 Schematic diagram of the protection mechanism of PEG.

It was found that the presence of PEG on the surface of AF-HSPC:CH liposomes (AF-PEG-HSPC:CH) (**A**) resulted in a slightly longer lifespan in the circulation, as compared to liposomes without PEG (AF-HSPC:CH) (**B**). This supports a general belief that PEG increases the circulation half-life of liposomes. It is suggested that the unique structure of PEG repulses C3b and/or plasma proteins and results in the decreased opsonization in plasma (**A**). By this means, PEG-containing liposomes escape from the capture of the MPS and have a longer lifespan.


KEEP GOING & GOING ......
PEG*
C3b
A
H2O
C3b Receptors
Plasma Proteins
Macrophages
MΦ
AF
PEG*


C3b Receptors
AF
I AM DEAD !! ......
C3b
Macrophages
MΦ
Plasma Proteins
MΦ
MΦ
B

V. CONCLUSIONS AND FUTURE DIRECTIONS

In summary, this study shows that AF-liposomes are better recognized by ß-galactose receptors than $ASGM_1$-liposomes or LAC-liposomes *in vitro*, mainly due to the likelihood that the AF-biotin-avidin complex on the AF-liposome surface is more available to ß-galactose receptors on hepatoma cells (with or without transfection of HBV), and the tri-antennary structure of AF with a terminal galactosyl residue on each branch, offers a better opportunity for multivalent binding which is essential for a stable binding to ß-galactose receptors (Baenziger and Maynard, 1980). It is clear that liposomes, including AF-liposomes, are good carriers for ddGTP. The basic liposome composition used in our investigation (HSPC:CH, 2:1) prevents the leakage of ddGTP from liposomes, due to the incorporation of the rigid phospholipid HSPC, which is below its liquid-crystalline phase transition at physiological temperatures. This results in a lower leakage of ddGTP from HSPC:CH liposomes at physiological temperature, as compared to liposomes composed of fluid phospholipids, e.g., egg PC. Incorporation of cholesterol into liposomes may also contribute to the decreased leakage of ddGTP from liposomes composed of HSPC:CH. AF-liposomes and plain liposomes appear to be internalized by 2.2.15 cells, and the internalization of AF-liposomes is through an uptake, mediated by ß-galactose receptors, in which ddGTP, encapsulated in AF-liposomes, is taken into lysosomes. However, an attempt to use AF-liposomes containing ddGTP to inhibit viral replication in 2.2.15 cells failed, possibly because of the inactivation of ddGTP by acid phosphatase in lysosomes (Barrett, 1984; Arsenis et al., 1970; Meijer et al., 1992). If this were to happen, 2.2.15 cells lack the kinase required to reverse the action of the acid phosphatase (Kitos, 1994) and reconvert ddG into ddGTP, its active form. The two-fold higher uptake of AF-liposomes, by the hepatoma cell line, over that of the HBV-transfected hepatoma cell line (Fig. 7B), indicates a possible down regulation of surface ß-galactose receptors on hepatoma cells due to HBV infection. The difference between the uptake of plain liposomes by HBV-transfected and non-transfected hepatoma cells (Fig. 1) suggests that HBV infection has altered other properties of hepatoma cells, possibly because HBV-infected hepatocytes may have altered receptor characteristics.

Targeting immunoliposomes against HBsAg resulted in a two-fold increase of uptake over plain liposomes on HBV-infected hepatoma cells (Fig. 10). Because of the difficulty of determining the timing of high levels of surface expression of HBsAg and difficulty in obtaining an mAb against the specific subtype of HBsAg presented on HBV-transfected hepatoma cells used in this study, it is difficult to optimize the binding between mAb-liposomes and the HBsAg on hepatoma cells transfected with HBV. A possible obstacle to the clinical use of mAb-liposomes against HBsAg is that, after injection, mAb-liposomes will bind mainly to the empty envelopes (abundant in the circulatory system of both acute HBV patients and syndrome-free carriers) competing for binding to HBsAg on the HBV-infected hepatocytes.

The results of the *in vivo* study reveal that it is possible to target ß-galactose receptors on hepatocytes by using AF-liposomes *in vivo* (Fig. 16A). However, AF-liposomes may be recognized *in vivo* by other receptors, besides the ß-galactose receptors on hepatocytes, and thus contribute to a decrease in the specific uptake of AF-liposomes by hepatocytes.

The addition of PEG into liposomes resulted in only slightly longer circulation half-lives *in vivo*, as compared to liposomes without PEG (Table 9). The slightly longer circulation half-lives of liposomes containing PEG probably resulted from an increased hydrophilicity of the liposome surface imparted by the PEG polymers. The bilayer rigidity and small size of HSPC:CH liposomes (with or without AF) and possible saturation of rapid removal processes for such liposomes at the injected dose employed, may result in their relatively long circulation half-lives and, thus, the difference in circulation times, between liposome with and without PEG, was not large, as seen in our *in vivo* study. Incorporation of PEG into liposomes could result in greater penetration of the liver sinusoids by the liposomes (a kinetic effect resulting from their longer circulation half-lives), leading to increased liposome uptake by hepatocytes. PEG may also mask the AF conjugated onto the liposomal surface of PEG-HSPC:CH liposomes and/or interfere with the multivalent binding between AF-PEG-HSPC:CH liposomes and ß-galactose receptors on hepatocytes. Either way, the recognition (or non-recognition) of AF by ß-galactose receptors on hepatocytes did not result in an increase in receptor-mediated uptake of AF-PEG-liposomes, in comparison to PEG-liposomes, by hepatocytes *in vivo*.

A number of lines of investigation can be proposed in order to solve some of the questions raised by the present studies. The main object of future studies is to formulate an immunoliposome (or immunovirosome) preparation which can act as a carrier of ddGTP, to prevent the intracellular degradation of ddGTP, and to specifically target liposome-entrapped ddGTP to HBV transfected hepatocytes. The following points are proposed as possible future studies:

(1) To investigate the possibility of targeting hepatitis B core antigen (HBcAg) on 2.2.15 cells by using liposomes conjugated with mAb against HBcAg (anti-HBcAg). Since mAb against HBsAg and asialofetuin (or other ligands of ß-galactose receptors) are not perfect ligands for targeting 2.2.15 cells or infected hepatocytes, the first priority of the future study is to discover a ligand specifically recognized by transfected hepatocytes. A possible candidate for this purpose is anti-HBcAg (Chen, 1993). HBcAg is expressed on the HBV-infected cell membranes and, unlike HBsAg or HBeAg, is not released from the membrane. Since the HBcAg is specifically located on the surface of HBV-infected cells and does not appear in the circulatory system, liposomes conjugated to anti-HBcAg may bind specifically to HBV-infected hepatocytes. One can consider using a coupling method other than the MPB-PE method used in this study to optimize the conjugation ratio of anti-HBcAg to phospholipid and possibly improve the uptake of such liposomes by 2.2.15 cells by preventing masking of the mAb by the PEG chains.

(2) To clarify the metabolic fate of ddGTP after liposomal delivery to hepatocytes, a high efficiency (or high pressure) liquid chromatography (HPLC) analysis can be used to quantitate the intracellular levels of ddG, ddGMP, ddGDP, and ddGTP after incubation of various liposomes (e.g., mAb-, AF- or plain liposomes) containing ddGTP with 2.2.15 cells. Depending on the results,

a more effective delivery system for ddGTP could be devised.

(3) To more precisely evaluate the antiviral effect of liposomal ddGTP by directly quantitating the radioactivity of the ^{32}P-probe hybridized to HBV-DNA fragments incorporated in cellular DNA from HBV-infected hepatocytes (or hepatoma cells). This could allow optimization of the dose and/or dosing schedule of liposome-entrapped ddGTP.

(4) To investigate the possibility of using virosomes or pH-sensitive liposomes (pH-liposomes) to prevent the lysosomal degradation of ddGTP by either direct introduction of ddGTP into the cell cytoplasm or the promotion of rapid release of the drug from the liposomal compartment. Virosomes or pH-liposomes may be loaded with ddGTP and incubated with 2.2.15 cells, and the fate of drug may be followed by HPLC analysis of intracellular ddG, ddGMP, ddGDP, and ddGTP levels.

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VII. APPENDIX

APPENDIX I

Buffers and Reagent Solutions

Appendix I

Denhardt's Reagent (50x stock)

ficoll (Type 400, Pharmacia) 0.01 g/ml

polyvinylpyrrolidone 0.01 g/ml

bovine serum albumin, BSA (Pentex, Fraction 5) 0.01 g/ml

filter and store small aliquots at -20°C

Fiske Sabbarow Reducing Reagent

sodium bisulfite (anhydrous) 15 g

sodium sulfite (anhydrous) 3 g

1,2,3-aminonaphthol sulfonic acid 0.25 g

in 125 ml distilled water

stand 3 hrs. in dark/filter into an Amber bottle

storage at 4°C/stable for 6-8 wks

Hank's Buffer, pH 7.35

8 g NaCl

0.4 g KCl

0.2 g $MgSO_4.7\ H_2O$

0.06 g $Na_2HPO_4.2\ H_2O$

(or 0.15 g $Na_2HPO_4O.\ 12\ H_2O$)

1 g glucose

2.4 g HEPES

in 1000 ml, adjust to pH 7.35 before use

Neutralizing Solution, pH 8.0

1.0 M Tris/HCl (pH 8)

1.5 M NaCl

sterilized by autoclaving; store a room temperature (RT)

Nick-translation Buffer (10x)

0.5 M Tris/HCl (pH 7.5)

0.1 M MgS04

0.001M DTT

500 μg/ml BSA (Fraction V; Sigma)

store in small aliquots at -20°C

PBS

0.137 M NaCl

2.68 mM KCl

10.6 mM Na_2HPO_4

1.47 mM KH_2PO_4

adjust to pH 7.4 at RT

Prehybridization Solution

25% formamide

5x SSPE

5x Denhardt's reagent

0.1% SDS (sodium dodecyl sulfate)

0.01 M Tris/HCl, pH 7.4

store at 4°C

20x SSC, pH 7

3.0 M NaCl

0.3 M sodium citrate

to pH 7 with NaOH or citric acid

sterilized by autoclaving

SSE solvent

4 parts ethyl acetate

1 part isopropanolol

2 parts H_2O

several cycles of vigorous shacking followed by settling

allow the layers to separate overnight

collect the top layer; store at RT

20x SSPE pH 7.4

3.0 M NaCl

0.2 M NaH_2PO_4

0.02 M EDTA

to pH 7.4 with NaOH

sterilized by autoclaving

TE Buffer pH 8

0.01 M Tris/HCl pH8

0.001 M EDTA (pH 8)

sterilized by autoclaving, store at RT

Tris-saturated Phenol

liquified phenol

0.1% 8-hydroxyquinoline

extract several times with an equal volume of 0.5 M Tris/HCl pH 8

extract several times with an equal volume of TE buffer

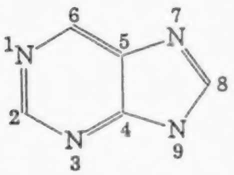
APPENDIX II

Chemical Structures of Nucleosides and Analogs

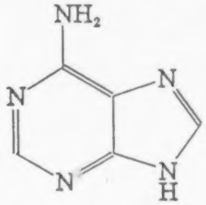
Appendix II

Physiological Purines and Pyrimidines: Chemical Structures and Numbering Systems

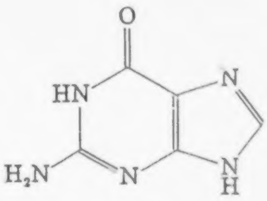
1. Bases
   a) Purines



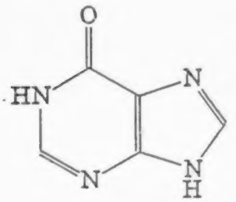
1) Numbering System



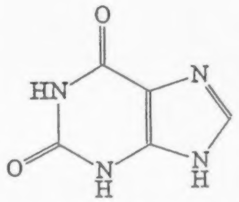
2) Adenine



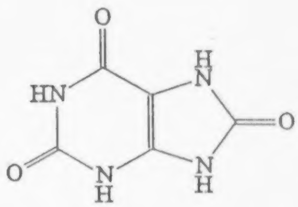
3) Guanine



4) Hypoxanthine

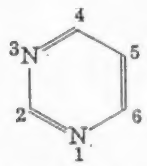


5) Xanthine

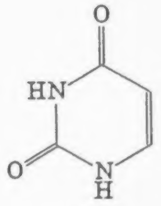


6) Uric Acid

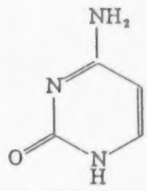
   b) Pyrimidines



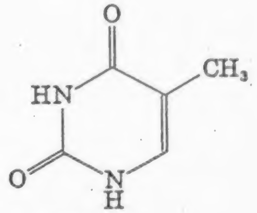
1) Numbering System



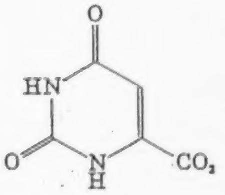
2) Uracil



3) Cytosine



4) Thymine

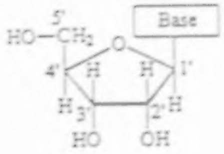


5) Orotate

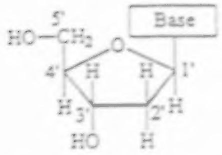
Appendix II, continued

2. Nucleosides

a) Numbering System

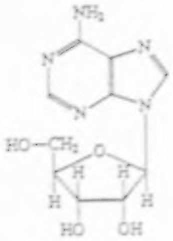


Ribonucleoside

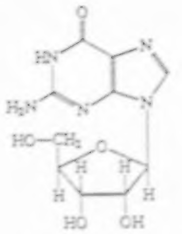


Deoxyribonucleoside

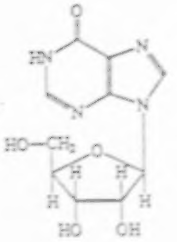
b) Purine Nucleosides



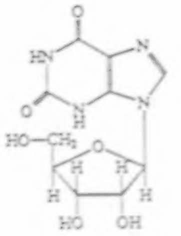
1) Adenosine



2) Guanosine

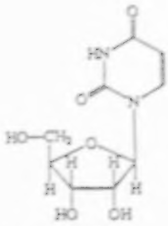


3) Inosine

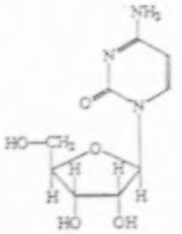


4) Xanthosine

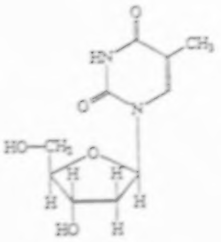
c) Pyrimidine Nucleosides



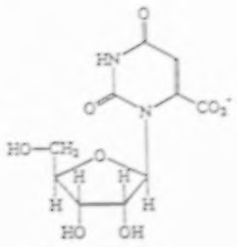
1) Uridine



2) Cytidine



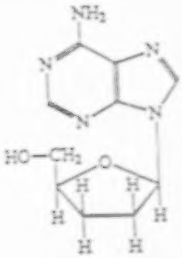
3) Thymidine



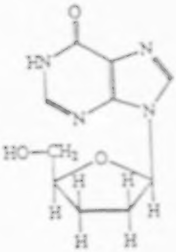
4) Orotidine

. Dideoxynucleosides

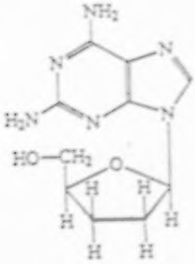
a) Purines



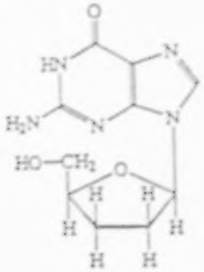
1) ddA
2',3'-dideoxyadenosine



2) ddI
2',3'-dideoxyinosine

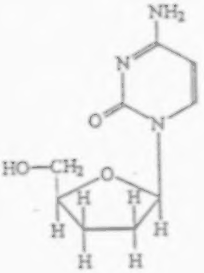


3) ddDAPR
2,6-diaminopurine 2',3'-dideoxyriboside

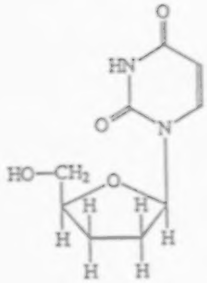


4) ddG
2',3'-dideoxyguanosine

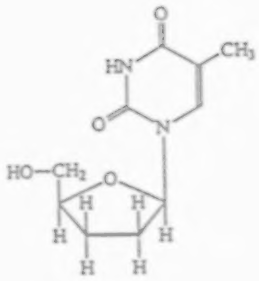
b) Pyrimidines



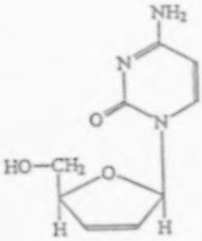
1) ddC
2',3'-dideoxycytidine



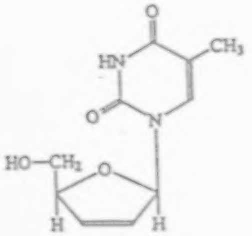
2) ddU
2',3'-dideoxyuridine



3) ddT
2',3'-dideoxythymidine

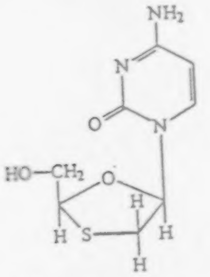


4) D4C
2',3'-didehydro-2',3'-dideoxycytidine



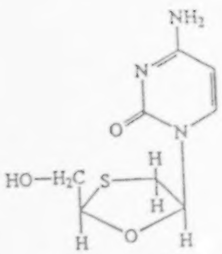
5) d4T
2',3'-didehydro-2',3'-dideoxythymidine

Heterocyclic Dideoxynucleosides



1) (+)BCH-189

2',3'-dideoxy-3'-thiacytidine



2) (-)BCH-189; 3TC

1-β-L-2',3'-dideoxy-3'-thiaribofuranosylcytosine

(NOTE: a racemic mixture of 1) and 2) is (±)SddC)